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14. ABSTRACT

In this Idea Award we proposed that a nutritional–immunological combination may be effective in stimulating the body's natural immune defenses against breast cancer. We hypothesized that retinoic acid (RA), a metabolite of vitamin A that has been shown to modulate the differentiation and/or activation of several types of immune cells, and α-galactosylceramide (α-GalCer), a synthetic lipid known to activate certain immune cells and exert antitumorigenic activity, will reduce the growth and/or stimulate natural immunity against 4T1 tumors in adult female Balb/C mice inoculated with 4T1 syngenic breast tumor cells. This report summarizes the final year's work. We have injected 4T1 breast tumor cells intravenously to simulate hematogenous diffusion and we have examined immune system response, micro-metastases, and the expression of Matrix Metalloproteinase (MMP) genes, MT-MMP1 and MMP3, that may be involved in the metastasis to the lungs. In vivo treatment with RA and αGalCer reduced the number of lung micrometastases ($P<0.05$), whereas each agent alone had an intermediate effect. The combination also reduced expression of MT-MMP-1 and MMP3 in lung, and in 4T1 cell supernatants. The overall results support the idea that a nutritional–immunological combination could be effective in reducing the growth and metastasis of breast tumor cells. The data point to reduced MMP production by the tumor cells as a possible new mechanism through which RA + αGalCer reduce tumor invasion and growth.

15. SUBJECT TERMS
Breast cancer cells; immune cell populations; retinoic acid; natural killer T cells; micro-metastases

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Introduction

This is a resubmission of a final report for the project: **A combined nutritional and immunological intervention to activate natural cytotoxicity against breast cancer cells in vitro and in vivo**. Year 3 of the project ended June 30, 2010. The project was continued from July 1, 2010 to June 30, 2011 under a no-cost extension to allow completion of aims and preparation of a manuscript. A manuscript resulting from this work has now been published in the journal *Nutrition and Cancer* (1). The publication is included as an attachment in the Appendix. This reports provides a detailed summary of the entire 3-year project.

The report previously submitted in Aug 2012 was disapproved for not being closely enough aligned with the SOW. In consultation with Dr. Patricia Green in 2012, I had prepared and submitted a modified SOW, which Dr. Green approved. In this revised Final Report our progress is reported against the Modified SOW. If the Modified SOW did not clearly state what the original task was (noted as "Completed"), then we have briefly restated the original task. We apologize for not having provided a clearer explanation previously. In retrospect, our original SOW had too many small tasks, some of which were simply administrative tasks, and we should have written our original application with fewer, broader tasks focused just on our research aims. This would have made reporting easier. For example, tasks 10-14 and tasks 15-18 should have been one task with multiple parts, and are now reported together. However, this was our first application for a Department of Defense research proposal we were naïve about how to best prepare an SOW. Some of our tasks were simply to prepare materials (e.g., tasks 1, 2, 15, 16), monitor mice, or to repeat and expand the number of observations. In this report, we have placed data where the research objective is described, and we have noted by cross-referencing where results from more than one task have been addressed. To summarize our original proposal, we had 3 aims: 1) To test in vitro the ability of RA and immune stimuli α-galactosylceramide (α-GalCer), in vivo on 4T1 murine breast cell growth; 2) To test in vivo the ability of transferred cells, pulsed in vitro with RA to induce CD1d and alpha-GalCer to bind to CD1d, to stimulate iNKT cells and inhibit the growth and metastasis of 4T1 murine breast carcinoma cells in a syngeneic mouse model (Balb/C mice); 3) To determine whether adding in vivo treatment, including poly-I:C, will be more effective than the simple ex vivo treatment with RA and α-GalCer tested in aim 2.

As explained in our modified SOW, the overall project goals did not change appreciably during the project but the approaches needed to be modified, and thus there were three shifts in direction: first, we shifted focus from 4T1 breast tumor cell growth in the mammary fat pad, where measurement of the tumor proved difficult, to the lung tissue where we noticed the development of micrometastases. Second, we eliminated the use of IFN-g knock-out mice in aims 1 and 2, however we added CD1d knock-out mice in aim 3. This was preferable for addressing our main interest in α-GalCer, which binds to CD1d and stimulates natural immunity. The use of CD1d knock out mice allowed us to conclude that the effect of α-GalCer was CD1d-dependent. Third, in aim 3 we conducted in vivo treatments with RA and α-GalCer as planned but replaced in vivo treatment with poly-I:C with an analysis of matrix metalloproteinase (MMP) gene expression in the RA and α-GalCer-treated mice, which had not been proposed originally but was reasonable based on the data we had obtained on lung metastasis. This became modified task 18.

Body

Hypothesis

We hypothesized that a combination of retinoic acid (RA), a metabolite of the essential nutrient vitamin A, which has immune stimulating properties, and alpha-galactosylceramide (αGalCer), a glycolipid known to alter immune function and to display antitumorigenic activity in vivo, could work together to stimulate the immune system against breast tumor cells. We proposed these agents may work more effectively together to stimulate the body's natural immune defenses against breast cancer. The conceptual model shown in Figure 1 was proposed. Our idea was based on *1)* previous work which has shown that αGalCer, a ligand for CD1d (2,3,4), which is an MHC-I like receptor, has potent antitumor effects against the growth of melanoma cells, lung cancer cells (5) and other tumors in several animal models; and *2)* our own published work showing that RA can induce the expression of CD1d on human and murine monocytic cells (6). We therefore considered that the combination might work in a synergistic manner to stimulate the body's immune defenses, including dendritic cells (DC) and natural killer (NK) cells against tumor cells in vivo (**Figure 1**). The reviewers of our idea considered it relatively high risk but novel, and noted that the strategy had potential for clinical application.

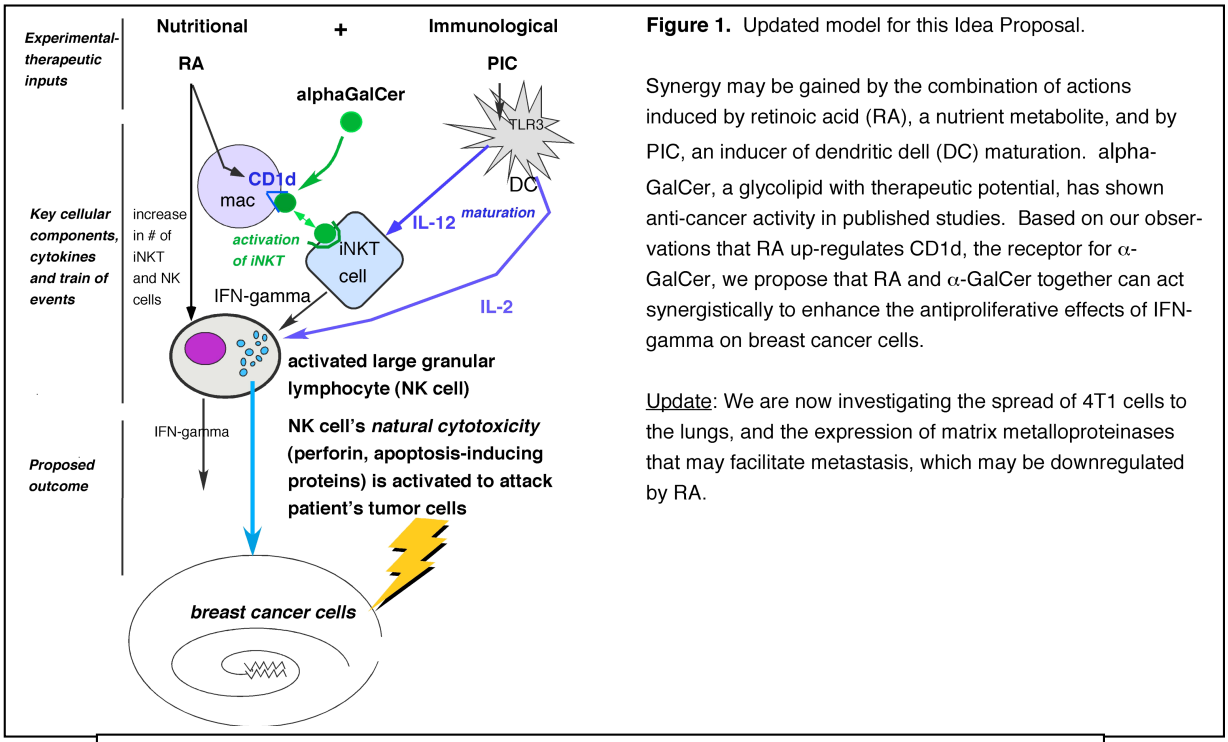

Figure 1. Updated model for this Idea Proposal.

Synergy may be gained by the combination of actions induced by retinoic acid (RA), a nutrient metabolite, and by PIC, an inducer of dendritic dell (DC) maturation. alpha-GalCer, a glycolipid with therapeutic potential, has shown anti-cancer activity in published studies. Based on our observations that RA up-regulates CD1d, the receptor for α-GalCer, we propose that RA and α-GalCer together can act synergistically to enhance the antiproliferative effects of IFN-gamma on breast cancer cells.

Update: We are now investigating the spread of 4T1 cells to the lungs, and the expression of matrix metalloproteinases that may facilitate metastasis, which may be downregulated by RA.

Figure 1. Working model for proposal.

Progress by task

Task 1: **Completed**. As stated in our original and modified SOWs, we obtained Institutional Animal Care and Use Committee approval for our studies. Approval was renewed after three years.

Task 2: Completed. We completed all regulatory compliance training and updated our Penn State worker protection courses (Radiation Safety; Animal Care). We obtained necessary materials and checked inventories of reagents. 4T1 breast tumor cells, which are syngeneic with Balb/C mice (7), were obtained from Dr. Danny Welch, University of Kansas, and were used throughout our studies.

Task 3. As stated in our Modified SOW**,** Aim 1 was to determine whether all-*trans*-RA, α-GalCer treated dendritic cells (DC) are able to reduce the growth of 4T1 tumor cells inoculated into the mammary fat pads of female Balb/C mice.

We completed task 3 by conducting two experiments, one of 26 days duration and a shorter 18-day study, using DC pulsed with RA and α-GalCer and 4T1 cells injected into the mammary fat pad. (As will be discussed, we revised this delivery protocol for later tasks.)

DC preparation*:* We began by preparing splenic DC as described by Toura et al. (5). For each preparation, cells were collected from two or three wild type (WT) donor female Balb/C mice. We distributed the cells in 24-well plates in complete medium (RPMI-1640 medium with 10% fetal bovine serum and antibiotics). Treatments of DC included: none (no additions except ethanol control); and RA, 100 nM in 0.1% ethanol, as tested in preliminary studies; plus pulsing with α-GalCer (Enzo Life Sciences), 100 ng/ml. We did not test separate treatments as it was more important to see if the combination was effective. The DC were treated overnight and collected just prior to intravenous administration to mice inoculated with 4T1 tumor cells.

Inoculation*:* Female Balb/c mice from Charles River were housed in plastic air-filtered cages with free access to water, and standard chew diet were used in an orthotopic model in which mice were inoculated with 2 x 10^4 4T1 cells in 100 μl of PBS, directly into the inguinal mammary fat pad. α-GalCer-pulsed DC were injected retro-orbitally. This was a small pilot study, with n=4 control mice, n=5 4T1 only mice, and n=6 4T1 pulsed DCs mice.

Monitoring: After inoculation, mice were monitored closely. We palpated at the mammary gland site of injection every week. We determined whether the 4T1 cells formed a solid tumor mass near the injection site. Once the tumors reached between 0.5-1.0 cm, initially estimated to be about 4-5 weeks after 4T1 cell inoculation but actually at 26 days, then all of the mice were sequentially euthanized with carbon dioxide. (If tumors exceed 1 cm then according to our IACUC requirements the mice must be euthanized.) The tumor nodules were carefully dissected and weighed. (Note that we discuss under task 4 why we did not use MRI.) The statistical significance of results was evaluated using ANOVA and *t*-tests, as appropriate, in Prism or SuperANOVA software.

Results: The results shown in Figure 2 illustrate intermediate data after this initial experiment. Palpable mammary tumors at the injection site were first felt about 2 weeks after 4T1 cell injection. At first these felt "sandy", palpable only as a slight roughness under the skin. At the

time of dissection on day 26, the mammary gland tumors in nearly all of the mice were well localized as tight nodule(s) without local invasion. We carefully dissected the tumors and other organs such as spleen, liver and lung. Body weight as well as liver and lung weight did not differ among the treatment groups; however, a marked difference was noted regarding spleen size and weight (* denotes $P<0.05$ between treatments by 1-way ANOVA). The statistical significance of results was evaluated using Prism or SuperANOVA software.

As shown in **Figure 2**A, 4T1 cell injection caused a significant enlargement of the spleen (Figure 2A). Splenomegaly in the 4T1 model has been reported previously (7). It is interesting to note that with the injection of RA-treated, αGalCer pulsed DCs, spleen weight was reduced although not completely to the control level. The same pattern was observed for tumor weight. After DC injection the weight of the local tumor was reduced, implying that growth was slowed by the DCs (Fig. 2B).

As there was variability regardless of treatment, we asked whether spleen weight and tumor size (weight in g) were correlated and the results in Fig. 2C show that there was a significant correlation (n=11 represents 4T1 and 4T1+DC, combined). We also measured colony counts for the lung metastases (Fig. 2D). (Panel D relates to task 5, evaluation of metastases, but it shown here because it is related to this experiment.) Although these results are from a very small study, encouraged us to consider this assay further in the rest of our experiments. They indicated that 4T1 cells were escaping from the site of inoculation. As most breast cancer deaths are the result of metastasis, we considered this result the most interesting and so focused on micrometastases for our future studies.

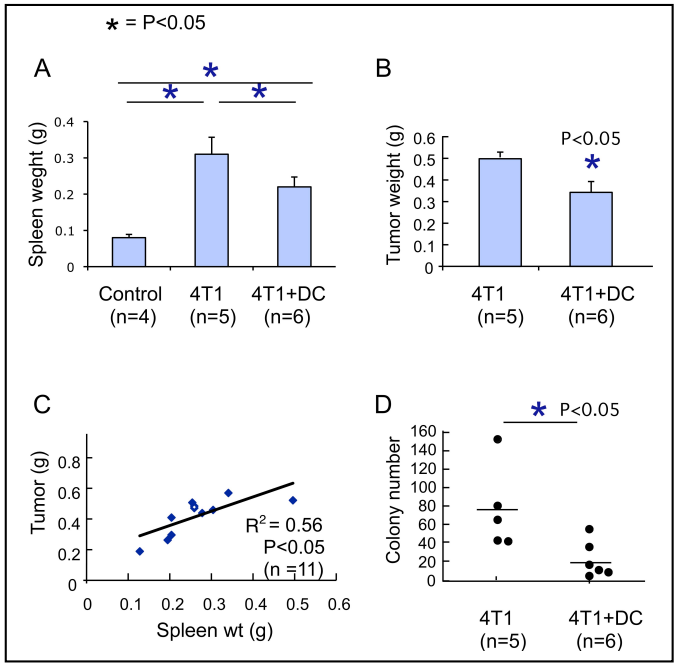

Figure 2. Spleen weight, mammary gland tumor weight and number of metastatic cells in lung of mice treated with RA-treated, αGalCer-pulsed DC and inoculated with 4T1 breast cancer cells.
A. Spleen weight (t-tests).
B. Tumor weight at mammary gland site (t-test);
C: Regression analysis;
D. Lung metastasis number (t-test). A, B, and D: $P<0.05$ by T-test.

Because the spleen in the mice bearing 4T1 tumor cells was quite large in the 26-day experiment (>3 fold weight increase) and the administration of DC normalized spleen weight and the spleen cell populations only partially, we performed a similar experiment for a shorter time, collecting tumors and spleen 18 days after inoculation with 4T1 cells and injection of DC. The size of the local tumor (mammary gland site) at the time of dissection was palpable but much smaller (about 2-3 mm in diameter or less) than in the 26-day experiment.

Results: This experiment was a pilot study to determine if a shorter time (18 days) could be used. We tested 4T1 cell administration, with DC that either were untreated, or treated with 20 nM RA, 100 nM of αGalCer, or both. Another group received no DC (*n*=4 per group). Spleen weight was highest in the absence of DC and lower in all groups with DC, regardless of treatment ex vivo (**Table 1**).

Table 1. Spleen and tumor weights in pilot study of DC treatment.

	n	Spleen wt, g Mean ± SEM	Tumor wt, g Mean ± SEM
Control (no tumor)	2	0.109± 0.010	None
4T1 with DC, Control treated	4	0.081 ± 0.011	0.026 ± 0.022
4T1 with DC, RA treated	4	0.093 ± 0.018	0.036 ± 0.024
4T1 with DC, aGalCer-treated	4	0.128 ± 0.017	0.067 ± 0.050
4 T1 with DC, both treatments	4	0.108 ± 0.009	0.019 ±0.010
4T1, no DC	4	0.148 ± 0.051	0.112 ± 0.041

Although these groups were too small for the differences to be significant by ANOVA, the tumor weights in the groups receiving DC treatments were all lower on average compared to the 4T1 group without DC. We therefore conducted an exploratory statistical analysis in which we pooled all the DC treatments, versus the 4T1 with no DC, and compared them as shown below.

Splenic enlargement caused by the 4T1 cells was still apparent (**Figure 3**), but the weight was only about half that in the 26-day experiment. Nevertheless, we again observed that injection of DC tended to decrease the splenomegaly caused by the 4T1 cells. Even though the mammary tumors were generally small in the 4T1 group, i.v. injection of DCs significantly decreased the local tumor growth in the mammary gland (no DC compared to all DC groups combined).

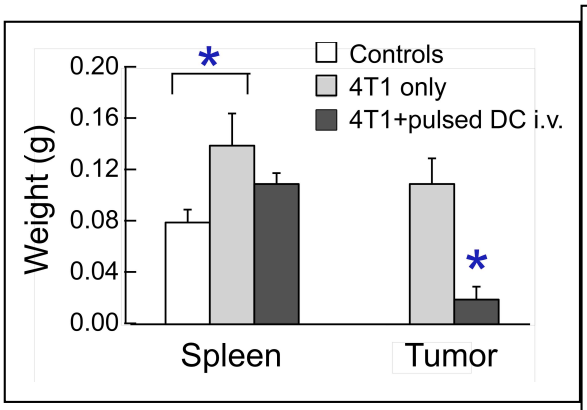

Figure 3. Spleen weight, tumor weight, splenic lymphocyte populations including macrophages and granulocytes, in mice inoculated in mammary fat pads with 4T1 cells and treated i.v. with pulsed DC (18 day pilot study).
A. Weights of spleen and tumor
(* notes $P<0.05$ as compared to control, by ANOVA or unpaired t-test).

(Other results from this study are shown with task 8).

In the 4T1 cell lung metastasis assay, although 4T1 cell colonies were detected in the 4T1-treated group, the colonies were much fewer than the previous 26-day study. The RA and α-GalCer-treated DC-injected group had no detectable colonies. Thus, it took a longer experiment to see the lung metastases when the tumor cells were introduced into the mammary glands, whereas by this time splenic enlargement was great. Later, we used the hematogenous route of delivery to directly, more quickly, and with more control, deliver 4T1 cells to the circulation so that they could seed the lungs and we could then test treatments with RA and α-GalCer in vivo.

Task 4. Completed. This aim of this task was to determine CD1d expression on isolated DC cells, and whether treatment with RA in vitro increases CD1d expression. For this experiment, bone marrow derived DC were prepared by flushing bone marrow from 2 Balb/C mice and culturing the collected cells for 9 days in the presence of GM-CSF (10 ng/ml). After this time the nonadherent cells (DCs) were collected and treated with RA (20 nM) for 48 hours. The surface CD1d expression was then detected using FITC-labeled anti-CD1d antibody (BD Biosciences).

Based on the forward/side scatter, DCs were gated in to A and B gates. Cells in the A gate, were shown to express a higher level of the maturation marker Ia antigen. These cells also had higher percentage of CD1d positive cells (**Table 2** and accompanying flow diagram).

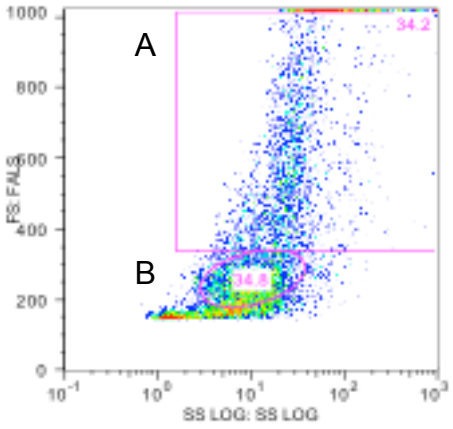

Table 2. Enrichment of CD1d expression on DC by RA

Treatment	% of gated cells in A gate	% of $CD1d^+$ Cells
Control	29.8 ± 1.9	7.04 ± 2.3
RA (20 nM)	30.3 ± 1.4	13.9 ± 2.0*

Mean ± SE is shown
$P = 0.0125$, RA compared to the Control by *t*-test.
Based on this result we expected that the RA-treated DC would be more capable of binding α-GalCer and thus becoming more active as APCs.

MRI. In our original proposal, we had proposed to use MRI for tumor detection. As this was not a task per se, there is no good place to discuss it. We include this explanation here so that it is clear why we changed our detection method (Table 3). In our original SOW, we had proposed to use a new MRI instrument at Penn State to image the tumor cells in vivo, a procedure that we thought would allow for multiple measurements over time. The Small Animal MRI facility had just opened and the methodology had not been applied previously for the purpose of detecting breast tumors. Using several mice injected with 4T1 breast cancer tumor cells we conducted MRI one week, and again 10 days and 14 days after tumor cell inoculation into the inguinal fat pads of otherwise untreated Balb/C mice. Initially, we could not visualize the tumor tissue by MRI, and even the distinction of major visceral organs was not as clear as we had hoped. The mice had to be anesthetized with isoflurane for a long period, up to an hour, to obtain good images and the duration of the anesthesia, which varied among mice, was a concern. After the tumors had grown to a palpable size, then they also were detectable by MRI but MRI did not offer an advantage for early detection. Since the purpose of using the MRI was to obtain early and sequential measures, we decided that the standard procedure of palpation and tumor measurement, using an accurate digital caliper that we had purchased, would be just as effective. Thus, the MRI part of the study was not as promising as we had hoped it would be. We therefore used conventional methods of tumor detection by palpation, caliper measurement and weight in our Modified SOW. Of these, weight was the most reliable because the tumors were not always round. Thus tumor weight is shown in the graphs in this report as a measure of tumor size.

Table 3. Summary of experience with MRI

Procedure	Days post-tumor injection	Mammary tumor detected?	Tumor was palpable	Comments
MRI	7 days	No	No	
MRI	10 days	No	Barely (sandy feeling under skin but not measurable with calipers)	
MRI	14 days	Just detectable	Detectable (1-3 mm)	Palpation and measurement with calipers was successful when MRI could detect tumors;
Palpation	14 days and later	Yes	Yes, measureable (3-5 mm range)	Anesthesia and expense of MRI were avoided.
Palpation/ caliper measurement /weight	Later than 14 days		Yes, up to 1 cm	

Since the purpose of using the MRI was to obtain early and sequential measures, we decided that the standard procedure of palpation and tumor measurement, using an accurate digital caliper that we purchased, would be just as effective.

Original tasks 5 and 6. Not initiated.

Explanation in our modified SOW: Original tasks 5-7 concerned measuring cell mediated toxicity and related cytokines. These tasks were not initiated because we did not have evidence for increased cytotoxicity due to the DC treatment in vivo. We had obtained preliminary evidence that lung metastases might be reduced and therefore we focused on this aspect, and on immune regulation, in **modified tasks 5-8**, below.

As noted in the modified SOW, **tasks 5-8** were revised to evaluate the lungs of mice treated with 4T1 cells given by orthotopic injection to mimic the hematogenous spread of tumors from the circulation into the lungs. Since the lung tumors were expected to be small we established a micro-metastasis assay, which was adapted from a published procedure by Du Pre et al. (8). In this assay, dispersed lung tissue is cultured in the presence of 6-thioquanine, which inhibits the growth of normal lung cells while allowing 4T1 cells to grow.

Modified task 5. Completed. We evaluated the lungs of mice administered 4T1 cells by orthotopic injection. For the orthotopic model, mice were inoculated with 2 x 10^4 4T1 cells in 100 μl PBS directly into the inguinal mammary fat pad. RA and α-GalCer-pulsed DC were injected retro-orbitally on the same days. Mice were closely monitored and once the tumors reached between 0.5-1.0 cm, about 28 days after 4T1 cell inoculation, all of the mice were sequentially euthanized with carbon dioxide, tumor nodules were dissected and weighed, lungs were subjected to histology and micrometastasis assay, and spleens were collected, weighed, and used for flow cytometry.

Lung tumors are expected to be small and therefore we used a micrometastasis assay. Lung tissues was minced and digested with 10 mg/ml of collagenase type IV and 10 U/ml of elastase for 30 minutes, and thereafter the cells were washed and cultured with medium containing 60 μM of 6-thioguanine. The colonies, formed after 7 to 10 days of culture, were counted after washing and fixation with methanol, and staining with 0.03% methylene blue dye, and counted. After counting, the dye was dissolved in 10% acetic acid and the absorbance at 570 nm measured to provide a second assessment of 4T1 cell growth. The latter method reflects both the number and the size of the micrometastases. Both methods agreed and OD570 was selected as the preferred method.

Results: Results for this assay are shown in Figure 4, and additional results will be shown in later figures. This task needed to be repeated with each experiment. These results suggested a decreased metastasis rate, or reduced growth once in the lungs.

Completed. Modified task 6. As our Modified SOW states we repeated this experiment for replication and to obtain a total of at least *n*=8 mice/group (with and without DC). We assessed the microarchitecture of the lungs by microscopy of paraffin sections stained with hematoxylin and eosin.

Results:

Hematoxylin and eosin (H&E) staining of the lung tissue showed that mice inoculated with 4T1 tumor cells but without any in vivo treatment with RA or α-GalCer after inoculation had multiple large tumor foci, while in mice that received these treatments the number and size of the foci were reduced. Figure 4 showed representative images from tumor-injected mice after 12

days after 4T1 injection without treatment (**Figure 4**, left) and with RA and α-GalCer treatment (Fig. 4, right).

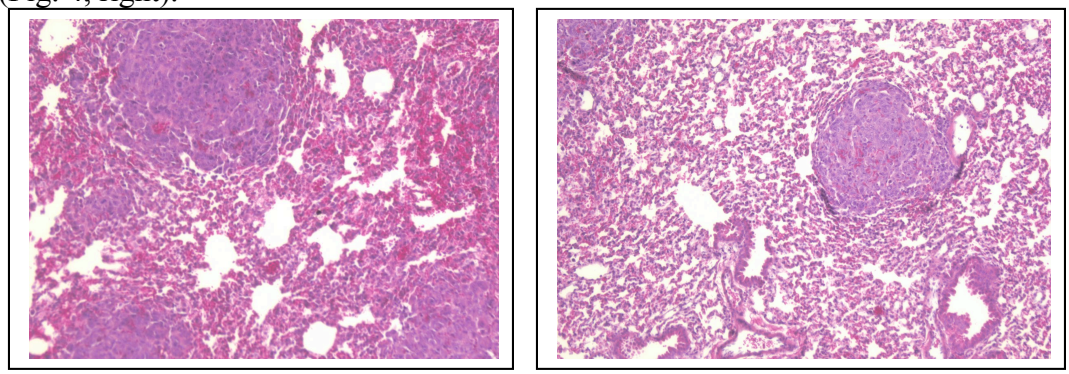

Fig. 4. H&E staining of mouse lung tissue. Left, Representative histology from 4T1 group without treatment. The field shows 4 metastatic growths and limited normal tissue; right, representative histology of 4T1 injected mice treated with RA and α-GalCer. The field shows one metastatic growth, with significant areas of normal alveolar structure.

To quantify the metastasis rate, lung tissue was digested with elastase and collagenase IV to isolate a single cell suspension. Cells were plated in 6-well plates to test the in vitro tumor foci formation. Cells were cultured with 6-thioguanine to eliminate normal lung cells, and after 14 days of culture the 4T1 cell colonies were counted. As shown in **Figure 5**, left, direct counting of the foci showed that all of the in vivo treatments had significant efficacy in reducing the number of micrometastases in the lung ($P<0.05$). The counting data were confirmed when we washed the plates and then dissolved the methyl blue dye used for detection and measured the optical density of the extracted dye at 570 nm (not shown).

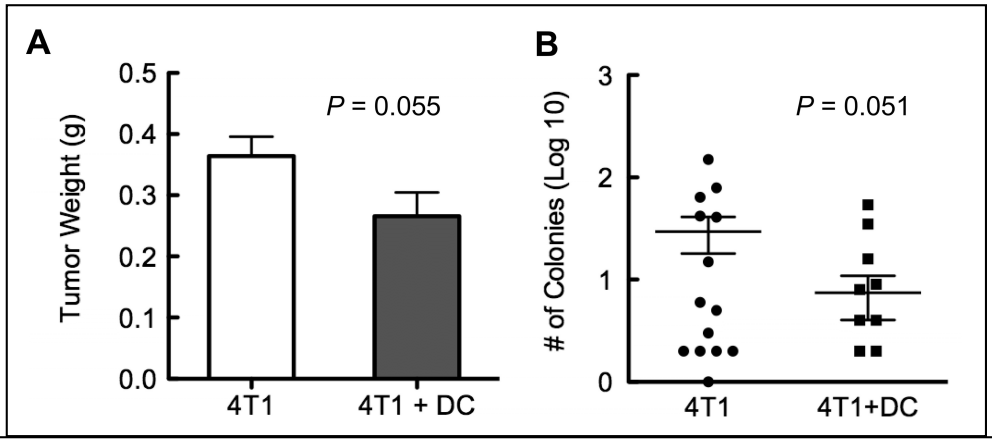

Figure 5. Tumor weight and # of micrometastatic colonies per lung in mice administered 4T 1 cells in the orthotopic model and treated with or without DC. Although results just barely missed being significant at the P<0.05 level they were considered very encouraging. Data are pooled from 3 independent experiments with *n*=4-6 mice/group in each experiment. Results were analyzed by *t*-test.

Not initiated: Original task 7. This task concerned measuring TNF and other cytokines produced by cytotoxic or NKT cells. Latter we measured MMP genes as being more relevant to the reduction in lung metastases we observed in this model. Thus although this task was not initiated at the time and sequence described, it was replaced by other experiments.

Not initiated. Original task 8. Original task 8 was to prepare a manuscript. We were not ready for this. We did submit an abstract to the Era of Hope meeting held in Baltimore. We continued with further studies conducted in modified tasks 7 and 8.

Completed. Modified Task 7. We next focused on immune activation, evaluating the effect of treatment on spleen enlargement and cell composition. It has been reported previously that, although the 4T1 cell model is a syngeneic breast tumor model, the spleen of the recipient mice showed enlargement and, thus, evidence of immune activation. To assess in our experiments whether treatment with DC reduces splenic enlargement, we determined spleen weight and morphology in tumor-bearing mice, with and without treatment with DCs. Spleen weight was compared to the weight of the tumor at the primary site to determine if there is a statistical correlation between tumor growth and splenic enlargement. From the studies in modified tasks 5-6 and a replication in task 7, we increased the sample size for correlation to n=22 mice. Linear regression analysis was performed using Prism (GraphPad) or SuperANOVA software, available in our laboratory.

Immune cell populations

Having observed splenic enlargement in this 26-day study, we analyzed the spleen for major cell populations as an indicator of immune activation. (Note, this is also related to task 8). Flow cytometry was conducted using well-characterized monoclonal antibodies that allow for determination of various cell types. Monocytic cells were detected using CD11b, with Gr-1 used for granulocytes, F4/80 for macrophages, and CD11c for DC. Lymphocyte populations were detected using CD3, CD4 and CD8 for total T cells and T-helper and T-cytotoxic cells, respectively, while natural killer T cells were detected using NK1.1 (antibody DX-5), and NKT cells by detection on the invariant T-cell receptor chain TCRß8. Antibodies to CD19 or B220 were used to quantify B cells, which are normally the largest population of splenic lymphocytes. The specific fluorescently tagged monoclonal antibodies (from BD Biosciences) that were used include CD19-PEcy7, CD3-FITC, CD4-PerCP, CD8-APC, NK1.1-PEcy7, CD11c-PEcy7, F4/80-APC, Gr-1-FITC, and CD11b-PE.

As shown in **Figure 6**A, the total T cell (CD3 positive) and B cell (CD19 positive) populations in the group of mice that received 4T1 cells only were reduced compared to the control groups without 4T1 cells, while the addition of the RA/α-GalCer-pulsed DCs tended to lessen the reduction in immune cells (no longer significantly lower in the 4T1+DC group). (For the statistical analysis we combined the control and DC only groups because neither had received tumor and the results were very similar, n=4. We compared this group to the 4T1 tumor group, n=5, and the 4T1+DC group, n=6.) The reduction in CD3 T cells was mainly in the CD8 T-cell compartment, similar in reduction to CD3 T cells in the 4T1 group, and partial reversed in the 4T1+DC group. No change was observed in the NK cell or NKT (Vß-TCR positive) cell populations. B cells were reduced by the tumor but treatment with DC did not correct this change (Fig. 6B). However, in contrast to the reduction in T cells by 4T1 cells, the granulocyte population was markedly increased in the 4T1 tumor group (Fig. 6B), with a trend towards reduction by treatment with DCs.

These data suggest that the 4T1 tumor cells induced changes in the spleen cell populations, with the reduction of T and B cells and increase of granulocytes. Co-injection of RA/α-GalCer pulsed-DCs could counteract some of the effects that the 4T1 cells had on splenic lymphocyte populations, which was apparently significant for the CD3 and CD8 (cytotoxic) T cell populations.

Since T cells and especially cytotoxic T cells are likely to be important in immune surveillance against tumors, we consider these results encouraging. A limitation is that the n/group was still small and we believed that effects might better be seen earlier in the course of tumor development. (It will be shown with task 8 that, after pooling data across several small experiments to increase the n/group, only CD3+ cells and B cells were significantly altered by 4T1 cell administration and DC treatment corrected only the CD3+ T cell results. CD11b+ and Gr1+ granulocytes were consistently elevated in the presence of 4T1 cells, regardless of treatment with DC.)

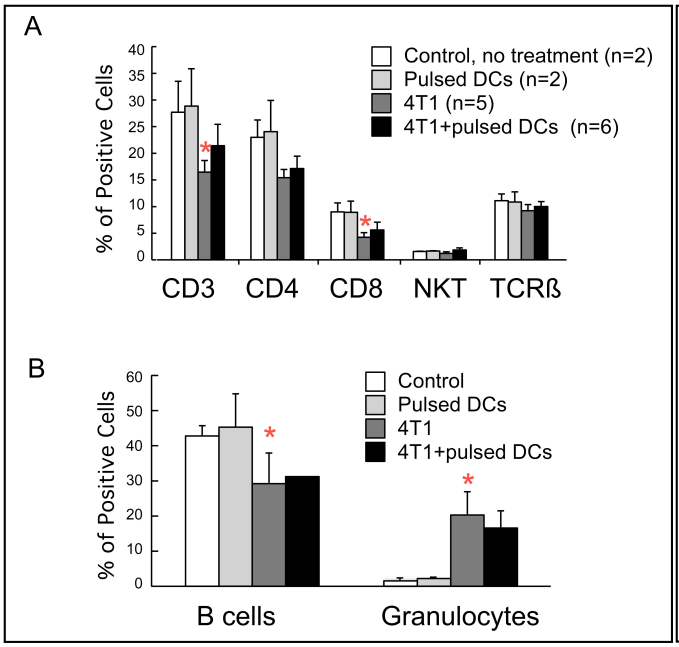

Figure 6. 4T1 tumor cells suppressed, and RA/α-GalCer -treated (pulsed) DCs partially restored the number of CD3 and CD8-positive cells in spleen (26 day study). Mean ± SEM; * indicates $P<0.05$ vs. combined controls. The antibodies used to identify each cell population are described in the text.

Splenic cells are also shown below in Figure 7 for another experiment in modified Task 8.

Completed. Modified task 8. A replication study was performed later in the first part of year 3, in a shorter-term 18-day study. Spleen weight, tumor weight and splenic cell composition were determined, similar to the study described above.

From the spleens in this study, we evaluated the impact of treatment on the immune system in terms of splenic immune cell composition. Similar to the study above, flow cytometry was conducted using well-characterized monoclonal antibodies that allow for determination of various cell types (see task 7).

In the 4T1 cell lung metastasis assay, although 4T1 cell colonies were detected in the 4T1-treated group (panel 7A), the colonies were much fewer than the previous 26-day study. The RA and α-GalCer-treated DC-injected group had no detectable colonies.

Similar to results in our 26-day experiment, 4T1 cells caused a reduction in the splenic T cell population, including NKT cells in the 18-day experiment (Fig. 7B), and significantly increased macrophages and granulocytes (Fig. 7C). However, the percentage increase due to the 4T1 tumor in 18-day study was less then the previous 26-day experiment. However, the pattern was similar with reduced CD3 T cells in the 4T1 group vs. a normal percentage in the 4T1 group that received the pulsed DC group. We did not observe a change in the proportion of B cells (about 45 to 50% in all of the groups, a normal percentage). B cells are the major white blood cell population in the spleen. It appears that the increase in the granulocyte and macrophage subset within the time span of this study probably was not enough to significantly alter/suppress the B cell population.

Consistent with the results above, CD3 T cells were reduced in the spleen of 4T1 tumor bearing mice, compared to both controls and mice that received α-GalCer pulsed DC. In this study we also observed a small but significant reduction in the 4T1 tumor bearing mice in the NKT cell population marked by the TCRß chain, although not when using DX5 antibody as a marker for total NKT cells (Figure 7A). This study also showed that mice given 4T1 cells, without pulsed DC, had an expansion of macrophages (F4/80$^+$ cells) and Gr1+ granulocytes (Fig. 7B).

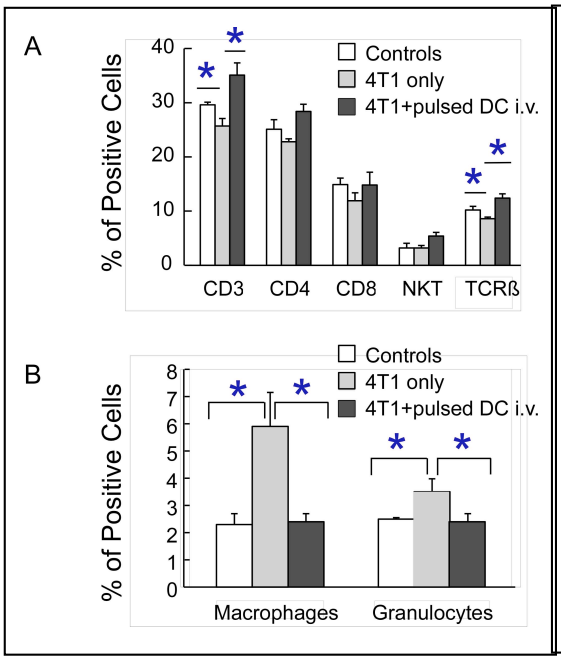

Figure 7. Splenic lymphocyte populations including macrophages and granulocytes, in mice inoculated in mammary fat pads with 4T1 cells and treated i.v. with pulsed DC (18 day study).
A: Flow cytometry with staining for T cells, CD4 positive T cells, CD8 positive T cells, NKT cells and iNKT cells detected by expression of the TCRVß8 chain.
B: Macrophages and granulocytes representing monocytic cells were detected.
* denotes differences between treatment groups, $P<0.05$.

Overall, these experiments suggested that 4T1 breast tumor cell injection could cause a systemic change in the immune system at an early stage, such as 18 days as shown above. The experiments demonstrated the effectiveness of pulsed-DC on the local tumor growth and lung metastasis.

For our publication, we pooled data from 3 independent experiments, some shown above, to make a final summation of the effect of 4T1 cells, in the orthotopic model (fat pad inoculation). These are shown in Figure 8. Spleen weight was significantly greater in the 4T1 group, and significantly reduced in the 4T1+DC group (A). For n=25 pairs (4T1 and 4T1+DC combined) the relationship of spleen weight to tumor weight was higher significant (B). Monocytes

(CD11b, shown in C) and granulocytes (Gr-1, shown in C) were both elevated in 4T1 mice regardless of DC, while CD11c+ DC were reduced in 4T1 mice regardless of DC treatment. These results were all highly significant, $P<0.01$. We also observed that CD3 positive T cells were reduced in the 4T1 group, although after all data were considered we no longer saw a correction in the 4T1+DC group. B cells (CD19+ were reduced in 4T1 mice regardless of DC. NK1.1 (NK cells) and TCRß positive (NKT) cells did not differ significantly.

It should also be noted that the cell analysis in task 8 was repeated in task 10 in which we used a hematogenous model of 4T1 cell administration. These results are shown with task 10.

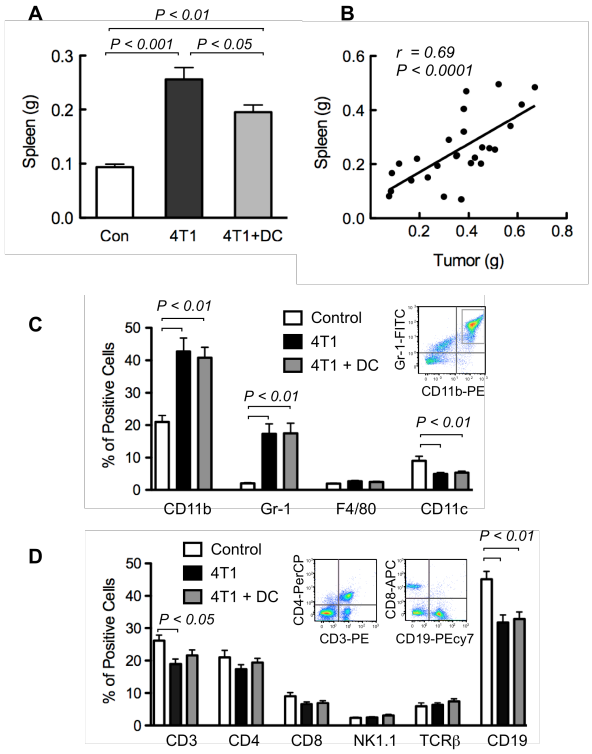

Figure 8. Splenic enlargement and cell population changes after 4T1 cell inoculation in Balb/c mice in the orthotopic model.
A. Spleen weight.
B. Correlation analysis of spleen weight and tumor nodules weight, $P < 0.0001$.
C. Staining for $CD11b^+$ cell population, most of which were $Gr\text{-}1^+$ cells in 4T1-inoculated mice. A representative scatter plot showing the population measured is shown in the upper left quadrant of the boxed panel.
D. Splenic lymphocytes were determined in Balb/c mice after 4T1 inoculation. Representative scatter plots are inserted to show the CD3, CD4, CD8 and CD19 staining and gating. Data were pooled from three independent experiments, with $n = 4$ to 6 mice/group in each experiment. Results of *t*-test are shown.

In modified task 8 we also conducted a parallel *ex vivo* experiment to determine if RA, α-GalCer, and both combined, are regulators of the production of growth factors by 4T1 cells. It has been reported that these cells autonomously produce granulocyte-monocyte colony stimulating factor (GM-CSF). One mechanism through which immune cells may be regulated is through this factor. We will assess GM-CSF expression in 4T1 cells cultured overnight with RA (20 nM), α-GalCer (100 nM), and both combined, on the expression level of GM-CSF and G-CSF mRNA, determined by quantitative PCR. The housekeeping gene HPRT was used as a reference and data were expressed relative to the control culture with placebo treatments only. The cells for each treatment were cultured in triplicate and the quantitative PCR assay was conducted in duplicate. The statistical significance of results was evaluated by ANOVA using Prism or SuperANOVA software.

Gene expression analysis

Because the expression of genes either by the tumor or surrounding tissue could be a factor in the growth of the 4T1 micrometastases, we screened MMP and chemokine genes as both gene families have been implicated in breast tumor growth.

Methods: We first conducted preliminary tests of gene expression using pooled mouse lung RNA. Other potential factors could be inflammatory chemokines (9). Using information in GeneBank, we designed primers for MMP2, MMP3, MMP9 and membrane type MTP-1, and CXCL1, CCL2, CCL3, CCL4, CLL5, and KLF13, and screened for their expression in pooled mouse lung tissue using quantitative real-time RT-PCR.

After selecting genes with a detectable level of expression (generally those detected in $\leq$ 26 PCR cycles) we assessed their expression by qPCR in the studies above using RNA prepared from lung tissue of individual mice.

To further support the data obtained for MMP3 by PCR, we determined the presence of MMP3 protein in plasma and cell supernatants using an ELISA assay. Since it is reasonable to hypothesize that MMPs may be secreted by the 4T1 cells, which thus promote the metastasis of these cells into the lungs, we also tested the ability of RA and α-GalCer to suppress MMP secretion by cultured 4T1 tumor cells, using culture supernatants in the ELISA assay. The data on gene expression measured directly in 4T1 cells, shown here, was part of task 20 (in vitro studies).

Results: As illustrated in **Figure 9**, MT1-MMP and MMP3 mRNA levels were significantly regulated by RA, α-GalCer and both in combination. The levels were increased in the lungs of mice with 4T1 tumor compared to tumor-free controls ($P<0.05$). Treatment with RA+α-GalCer brought the levels back down to that in control mice (both marked by "b" in panel A). Plasma levels of MMP3 protein were determined by a specific ELISA. Results for mice treated with the combination of RA+α-GalCer differed significantly from the group that received tumor and placebo, $P<0.05$. Other groups were slightly but not significantly reduced.

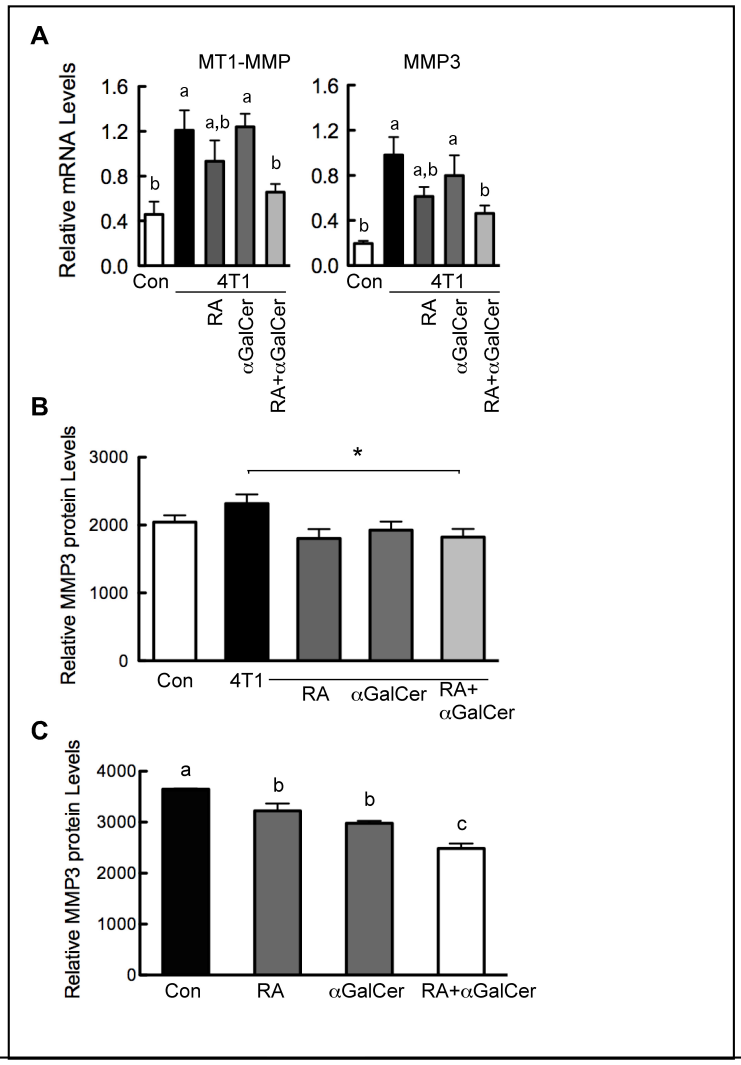

Figure 9. Matrix metalloproteinase expression levels in vivo and in cultured 4T1 cells. A. MT1-MMP and MMP3 mRNA levels were determined in lung tissue in control mice (Con) and it mice injected with 4T1 tumor cells ad treated with placebo (Black bar, no label) or with RA, αGalCer or RA+α-GalCer as described in the text. Data shown are the means and SEM for *n*=8-10 mice/group. Bars with different superscripts differed significantly, $P<0.05$. The results show that RA partially and the combination of RA+αGalCer significantly reduced MT1-MMP expression, back to the level in control mice without tumor. Similar results were observed for MMP3. B. MMP3 protein in mouse plasma was determined by an MMP3-specific ELISA. Data shown are the means and SEM for n=8-10 mice/group. Results for mice treated with the combination of RA+αGalCer differed significantly from the group that received tumor and placebo, $P<0.05$. Other groups were slightly but not significantly reduced. C. MMP3 protein level were determined by ELISA in the culture supernatant of 4T1 cells cultured in the presence and absence of RA (20 nM) and α-GalCer (100 ng/ml) for 24 hours. Data shown are the means and SEM for n=3 cultures/group. Bars with different superscripts differed significantly, $P<0.05$. Both RA and α-GalCer alone significantly reduced MMP3 production and secretion ($P<0.05$ versus Con), while RA+α-GalCer produced an additive decrease that was greater than for either RA or α-GalCer alone.

Our modified SOW next covered tasks 9 and 10 and commented on milestones we had initially set. In retrospect, we should not have called task 9 or 10 a task as they were not research objectives per se, just preparation for the next steps. However, we completed these preparations (also listed as Milestone 4).

Task 9: This goal of this task was to prepare for aims 2 and 3.

Completed: Milestone 2. We completed the first set of in vivo studies, with our modified emphasis on 4T1 tumor metastasis to the lungs. Immune activation was added due to having observed greatly increased spleen weight in 4T1 tumor-bearing mice. We also evaluated whether the treatments have a direct impact on the ability of 4T1 cells in culture to produce GM-CSF or G-CSF, as shown above.

Not initiated. Milestone 3. We were not ready to submit a manuscript from data in aim 1 and we realize in retrospect that the in vitro data alone, as we had originally proposed, would not have been adequate for a full manuscript.

Completed. Milestone 4. Technical preparation for both aims 2 and 3 was completed. Staff were trained in all aspects of work.

Completed: Task 10. This task was to begin in vivo studies (direct administration of α-GalCer) in aim 2. We initiated this, in slightly modified form, described below. It should be noted that the analytical method using MRI, which had been described as task 11, was changed to micrometastasis detection and incorporated into task 10.

Explanation: A new subtask was added because we needed to first obtain IACUC permission for these studies. We requested to use the hematogenous delivery model published by Kim EJ, et al. (11). We wrote a modification of our IACUC approval to permit use of this route of 4T1 cell administration. Additionally, although direct in vivo treatment was previously part of aim 3, it was moved up to aim 2 after we found that DC treated ex vivo with RA and α-GalCer and were not more effective than control DC. We therefore moved ahead to test the **direct in vivo** treatment of mice treated with RA and α-GalCer or both in combination. We redesigned aim 2 to examine 4T1 cell growth in the **lungs** of mice treated with RA and α-GalCer in vivo. We reduced the time of the study to 10-14 days (compared to 4-5 weeks with orthotopic tumor administration) to optimize evaluation of cell uptake and micrometastasis in the lungs.

A**im 2**. To modify the tumor model in order to focus on the effects of RA and α-GalCer given directly to mice in vivo as a means to reduce the growth of 4T1 cells in the lungs.

Experiment: We next investigated whether RA and α-GalCer alter tumor cell metastasis in mice without treatment with DCs. Mice were inoculated with 4T1 cells directly into the blood stream as a model of the hematogenous spread of tumor cells; this eliminates the step in which tumor cells must escape from the primary tumor, which is variable in timing and number of cells. Two x 10^4 4T1 cells in 100 μl PBS were injected directly into the circulation. Twenty-four hours later, mice were randomized to receive control (vehicle as canola oil), RA, α-GalCer, or both. All-*trans*-RA, 37.5 μg per mouse in canola oil, was given orally once a day for 10 days. α-GalCer, 2 μg per mouse in sterile saline, was given subcutaneously every other day. After 14 days, 4T1 cells in lung tissue were evaluated. Lung metastases were determined as described above, and splenic myeloid and lymphoid cells were determined (repeating task 8 in this new model).

Results: With this procedure, the weight of the spleen did not increase nearly as greatly as in the previous experiment (data not shown), which likely reflects the shorter time of this experiment since the number of tumor cell inoculated was the same for both this and our first experiment. However, spleen cell populations were still altered, with decreased lymphocytes and increased $CD11b^+$ cells, most of which were $Gr\text{-}1^+$ cells (**Figure 10**A,B). Treatment with RA and/or α-GalCer did not alter the population changes that were caused by the 4T1 tumor cells.

Either RA or α-GalCer alone reduced the rate of lung metastasis nonsignficantly, determined both by counting the lung tissue colony number and staining the colonies. However, RA+α-GalCer combined decreased lung metastasis significantly ($P<0.05$, Fig. 10C and 10D). Although the differences for RA and α-GalCer alone were not statistically different, the mean values were both lower. Thus it appears that with a larger sample (these results represent 15-17 mice/group), these single treatments might be effective. However, our hypothesis was that the combined treatment would be effective and these results differed significantly, $P<0.001$ by colony count and $P<0.01$ by dye elution assay.

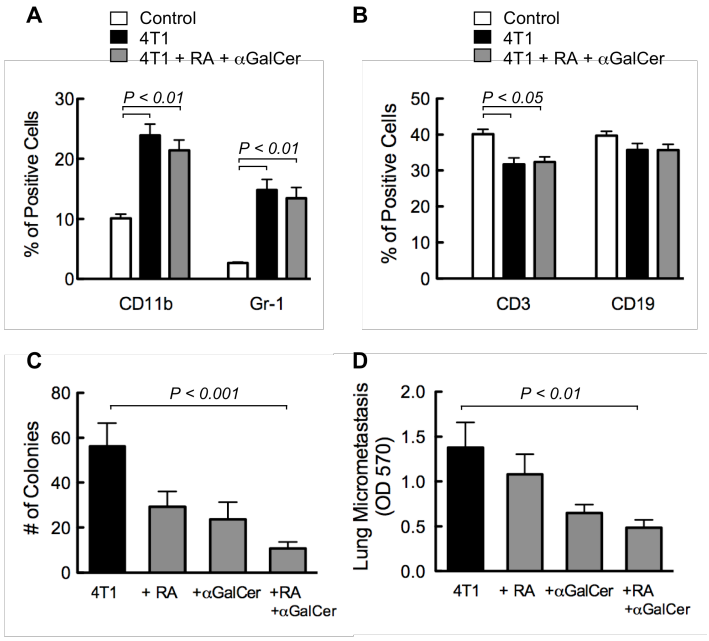

Figure 10. RA and α-GalCer decreased lung metastasis in a hematogenous model of 4T1 cell inoculation.
A. Spleen myeloid cells were determined by flow cytometry.
B. Lymphocytes were determined by flow cytometry.
C. Micrometastasis was determined by counting the number of colonies on each culture plate.
D. Micrometastasis was determined by measuring the absorbance at 570 nm, after crystal violet staining, which represents size and number of colonies. Data were combined from three independent experiments with $n = 5$ or 6 mice in each experiment. P values were determined by ANOVA.

Completed: Modified Task 11. Our original task 11 was to assess tumors in aim 2 by MRI. As explained in task 10, we assessed using micrometastases, as explained earlier, and splenic involvement; these results have already been shown with task 10.

Completed. Original task 12 was to assess histology and evaluate the data. This was already accomplished as part of task 10 by the measurement of micrometastases and the statistical analysis of the data.

Completed. Original task 13 was to repeat tasks 10-12 to increase sample size to n=5-6/group, completing aim 2**).** We did this as described in task 10.

Completed. Milestone 5: Completion of modified aim 2.

Completed. Original task 14. The original task was to complete analysis of all data for aim 2 and organize the results. We completed this and began to write a manuscript on how in vitro-pulsed cells decrease growth of breast tumor cells in vivo. However, we realized that it was important to determine the involvement of CD1d in this process. Therefore we added additional in vivo experiments, similar to those in tasks 11-13, to compare WT and CD1d knock out mice. These were addressed in modified tasks 15-18, below.

Completed with modifications: Original tasks 15-18. Original task 15 was to grow the cells and order mice, and task 16 was the actual treatment. Task 17 was to monitor the mice and task 18 was to analyze the tumors. Thus, tasks 15-18 are all part of a single objective. These tasks corresponded to aim 3, described as an in vivo study designed to compare DC-pulsed cells and direct treatment of mice with all-*trans*-RA, given orally, with α-GalCer. In this set of tasks we compared CD1d wild-type (WT) and CD1d "knock out" mice, as explained below. We have grouped the data for these tasks as all being part of task 18. Because it is well known that the MMP genes play important roles in tumor invasion and metastasis, analysis of MMP expression was added to the Modified SOW as part of task 18.

Completed: Original task 17. Treatments were monitored using methods described in tasks 5 and 8.

Completed: Modified task 18. Tissues were processed for micrometastasis assay.

Accomplishments for tasks 15-18 are addressed together here.

Explanation: In our studies so far it was not clear whether the effect of α-GalCer actually required the presence of CD1d. We took advantage of the availability of CD1d knock out mice from the Jackson Laboratory to determine whether CD1d, a receptor for α-GalCer, is essential for the effect we had observed for RA plus α-GalCer in vivo. Because studies using CD1d knock out mice would be expensive, and because we had observed that the combined treatment was more effective than either one alone, we redesigned this study to compare combined treatment vs. control, omitting treatments with RA and α-GalCer alone. We used age-matched female Balb/c WT mice from the same vendor as controls. Because only a few CD1d knock out mice could be delivered at a time, we found it necessary to conduct the study as three small experiments, each of the same design, from which the data could be pooled to obtain n=8-10 mice per genotype. The data shown are the pooled results.

Tumor metastasis and immune cell populations

Experiment: To further study the mechanisms of RA and α-GalCer in the prevention of tumor metastasis, we employed CD1d null mice to determine whether the effect of RA+α-GalCer is CD1d dependent. 4T1 cells were inoculated intravenously in both WT and CD1d-null strains. Treatments with all-*trans*-RA, 37 μg per mouse in canola oil, were given orally once a day for 10-14 days (monitoring animal weight as an indicator of when to terminate the study). Mice were also treated with α-GalCer, 2 μg per mouse, given subcutaneously every other day. The lung metastasis rate was determined as described above using crystal violet staining and cell counts. Spleen weight and cells types were assessed using the methods described above in tasks 7 and 8.

Results: After treatment with RA+α-GalCer, tumor metastases were lower in WT mice, but not in CD1d-null mice (**Figure 11A**, where OD570 represents the tumor micrometastases). Both WT and CD1d-null mice showed a similar marked increase in $CD11b^+$ cells and, to a lesser degree, a decrease in lymphocytes, especially B cells. CD1d-null mice treated with RA+α-GalCer had slightly higher percentages of $CD11b^+Gr\text{-}1^+$ cells and lower B cells, suggesting an altered cellular response (Figure 11B and C).

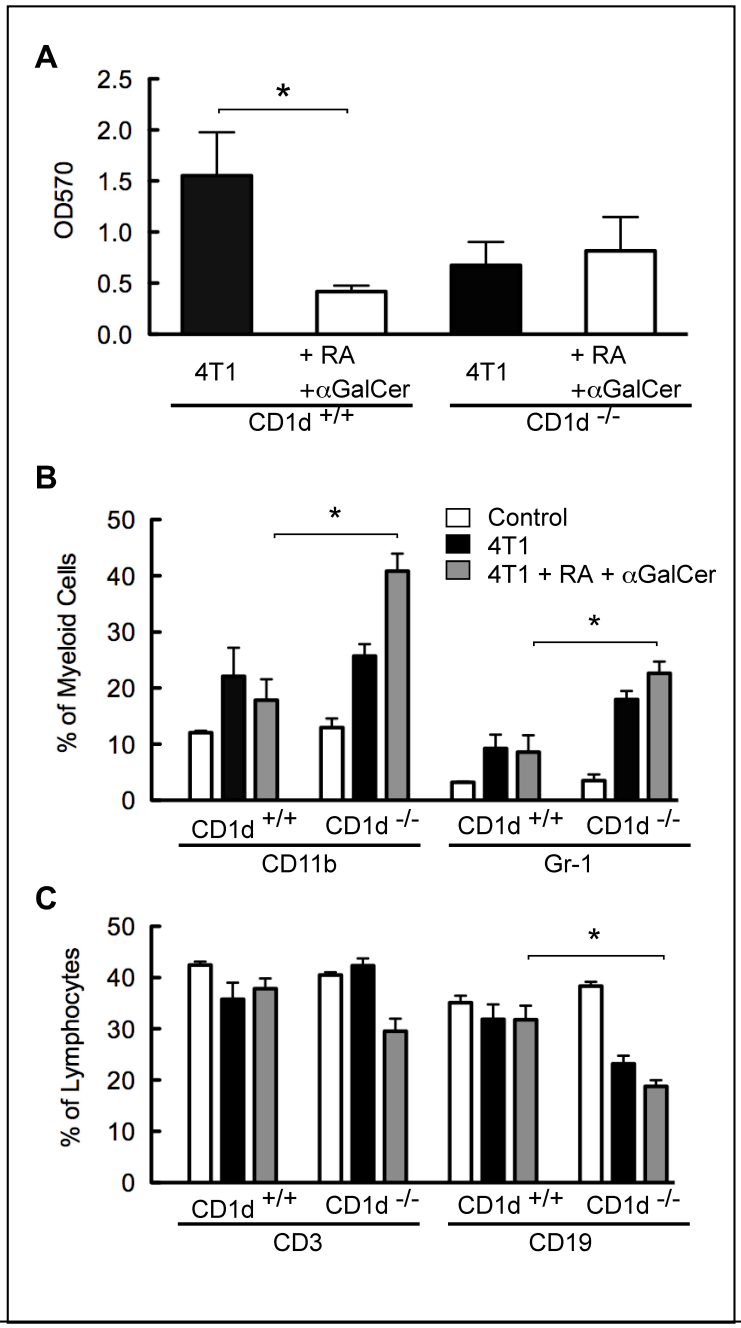

Figure 11. 4T1 tumor metastasis and immune cells in wild type CD1d+/+ and CD1d$^{-/-}$ mice. A. Lung metastasis rate represented by the absorbance at 570 nm are shown. The data shown were combined from two independent experiments with n = 5 or 6 mice in each experiment for a total of 10=12 mice/group.

A. In WT mice, RA+α-GalCer significantly reduced micrometastases compared to 4T1-bearing mice without treatment (*, P<0.05). In CD1d-/- mice, there was no difference between 4T1-bearing mice without and with RA+α-GalCer.

B. Splenic myeloid cell population.

C. Lymphocyte staining. The data shown were combined from two independent experiments with n = 5 or 6 mice in each experiment.

The results of this study were informative in showing that CD1d is essential for the reduction in lung 4T1 cell micrometastases due to treatment with RA+αGalCer. The results were somewhat complicated by the fact that tumor micrometastases were moderately (not significantly) lower in the CD1d-/- mice compared to WT mice regardless of treatment. We hope that in the future it will be possible to repeat this study with a larger sample size. If the CD1d-/- mice could be bred in house, instead of purchased as was necessary for our studies, the experiment might be less expensive and a larger number of animals could be studied.

Thus, having observed reduced metastases, we explored possible mechanisms. In modified task 18, we conducted preliminary tests of gene expression using pooled mouse lung RNA. Matrix metalloproteinases (MMP) are a family of zinc-dependent endopeptidases capable of degrading the extracellular matrix, which play important roles in tumor invasion and metastasis (10). Other potential factors could be chemokines. Using information in GeneBank, we designed primers for matrix metalloproteinases (MMP)2, MMP3, MMP9 and membrane type MTP-1, and CXCL1, CCL2, CCL3, CCL4, CLL5, and KLF13, and screened for their expression in pooled mouse lung tissue using quantitative real-time RT-PCR (Supplemental table S1 in Appendix). These genes were selected for analysis in the lung tissue of mice 12 days after 4T1 cell inoculation and treatment with RA, α-GalCer, or both in combination.

To further support the data obtained for MMP3 by PCR, we determined the presence of MMP3 protein in plasma and cell supernatants using a commercial ELISA assay for mouse MMP3 (Abcam, Cambridge, MA) according to manufacturer's instructions. Since it was reasonable to hypothesize that MMPs may be secreted by the 4T1 cells, which thus promote the metastasis of these cells into the lungs, we also tested the ability of RA and α-GalCer to suppress MMP secretion by cultured 4T1 tumor cells, using culture supernatants in the ELISA assay.

Results: We first screened the gene expression levels of several MMPs in lung tissue by quantitative real-time RT-PCR (Supplemental table S1 in Appendix). Among the several MMPs reported in the literature, the inoculation of mice with 4T1 cells more than doubled the gene expression levels of membrane-type 1 MMP (MT1-MMP) and MMP3 (**Figure 12A**). The lungs of mice with 4T1 cells had higher levels of expression of MT-MMP1 and MMP3 mRNA, indicating that the tumor alone activated the expression of these genes. This would be consistent with tumor-induced promotion of metastasis. As also shown in Figure 12, treatment with RA significantly decreased the levels of MMP3, with a similar tendency for MT-MMP1. α-GalCer did not affect gene expression, nor did it modify the effect of RA in the combination treatment. The lower levels of MMPs could help to explain the reduced metastasis rate in RA-treated animals. We then assessed MT1-MMP and MMP3 mRNA levels in the lungs of mice with and without treatment with RA and α-GalCer. Mice treated with RA and α-GalCer showed significantly decreased levels of both MT1-MMP and MMP3 mRNA in lung. To further examine MMP expression, MMP3 protein was measured in plasma. As shown in Figure 12B, the level of MMP3 in plasma was marginally increased in 4T1 tumor bearing mice, while it was reduced by treatment with RA+α-GalCer. MMP3 protein in 4T1 cell culture supernatant was also lower in mice treated with either RA or α-GalCer alone, as well as both in combination (Figure 12C). These data suggest that inhibition of MMP production by 4T1 tumors may be one of the mechanisms by which RA and α-GalCer can reduce the rate of lung tumor metastatic growth.

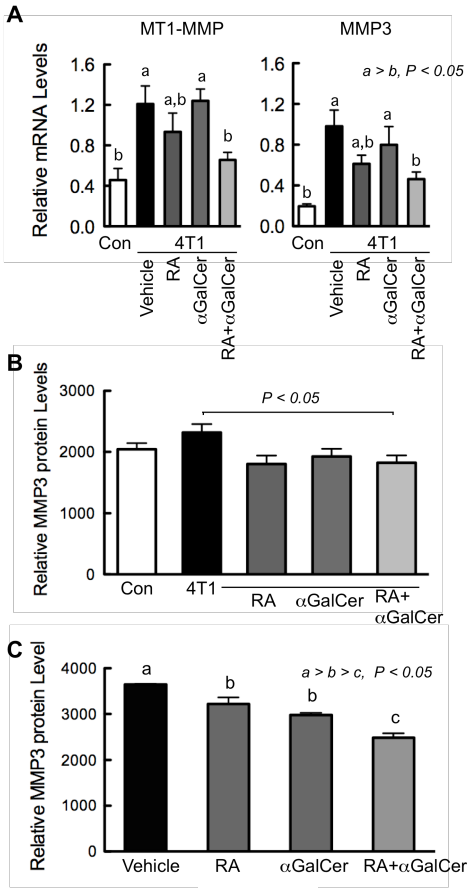

Figure 12. Matrix metalloproteinase expression levels in vivo and in cultured 4T1 cells.
A. MT1-MMP and MMP3 mRNA levels were determined in lung tissue in control mice (Con) and it mice injected with 4T1 tumor cells ad treated with placebo (black bar, no label) or with RA, αGalCer or RA+α-GalCer as described in the text. Data shown are the means and SEM for n= 8-10 mice/group. Bars with different superscripts differed significantly, P<0.05. The results show that RA partially and the combination of RA+αGalCer significantly reduced MT1-MMP expression, back to the level in control mice without tumor. Similar results were observed for MMP3.
B. MMP3 protein in mouse plasma was determined by an MMP3-specific ELISA. Data shown are the means and SEM for *n*=5-12 mice/group. Results for mice treated with the combination of RA+αGalCer differed significantly from the group that received tumor and placebo, *P*<0.05. Other groups were slightly but not significantly reduced.
C. MMP3 protein level were determined by ELISA in the culture supernatant of 4T1 cells cultured in the presence and absence of RA (20 nM) and α-GalCer (100 ng/ml) for 24 hours. Data shown are the means and SEM for n=3 cultures/group. Bars with different superscripts differed significantly, *P*<0.05 (ANOVA). Both RA and α-GalCer alone significantly reduced MMP3 production and secretion (*P*<0.05 versus Con), while RA+α-GalCer produced an additive decrease that was greater than for either RA or α-GalCer alone.

Overall, these results show that RA plus α-GalCer in vivo reduce MMP3 protein in lung tissue and plasma and its production and secretion by 4T1 cells. The conclusion is supported by a small but significant decrease in plasma MMP3 concentration for the combination of RA+α-GalCer. We could not measure plasma MT1-MMP because there no specific ELISA kit was available for it. The results for MMP3 protein in 4T1 cell supernatants suggests, first, that 4T1 cells are a source of MMP3, which could contribute to their ability to form metatastases, and also that the combination of RA+α-GalCer is more effective than either alone in decreasing the production and secretion of MMP3 from these tumor cells. These results suggest that MMP3 is a product of this breast tumor cell, and that its production can be down-regulated by RA and αGalCer. Further studies on the mechanism of the reduction would be of interest.

We also measured the proliferation and migration potential of 4T1 cells in culture to assess the direct effects of RA, α-GalCer and both combined on 4T1 cells, using ^{3}H-thymidine incorporation to test for a reduction in cell proliferation, and a filter assay to assess the ability of treatment to reduce the migration potential of 4T1 cells. 4T1 cells were cultured in the presence and absence of RA (20 nM) and α-GalCer (100 ng/ml) for 24 hours. For the cell proliferation assay, 1 μCi/well of ^{3}H-thymidine was added for the final 4 hours and the incorporated ^{3}H was measured by liquid scintillation counting after collecting cells on filter mats. For the migration assay, cells were cultured in filter plates and cells that had migrated through the filter were stained and the dye eluted and measured at 570 nm.

To determine if treatment with RA, α-GalCer and both combined have a direct effect on the expression of GM-CSF and G-CSF in cultured 4T1 cells, we determined the relative abundance of the mRNAs for these genes after treatments as described above. As shown in Figure 13, RA, but not α-GalCer alone, significantly increased the expression of both genes.

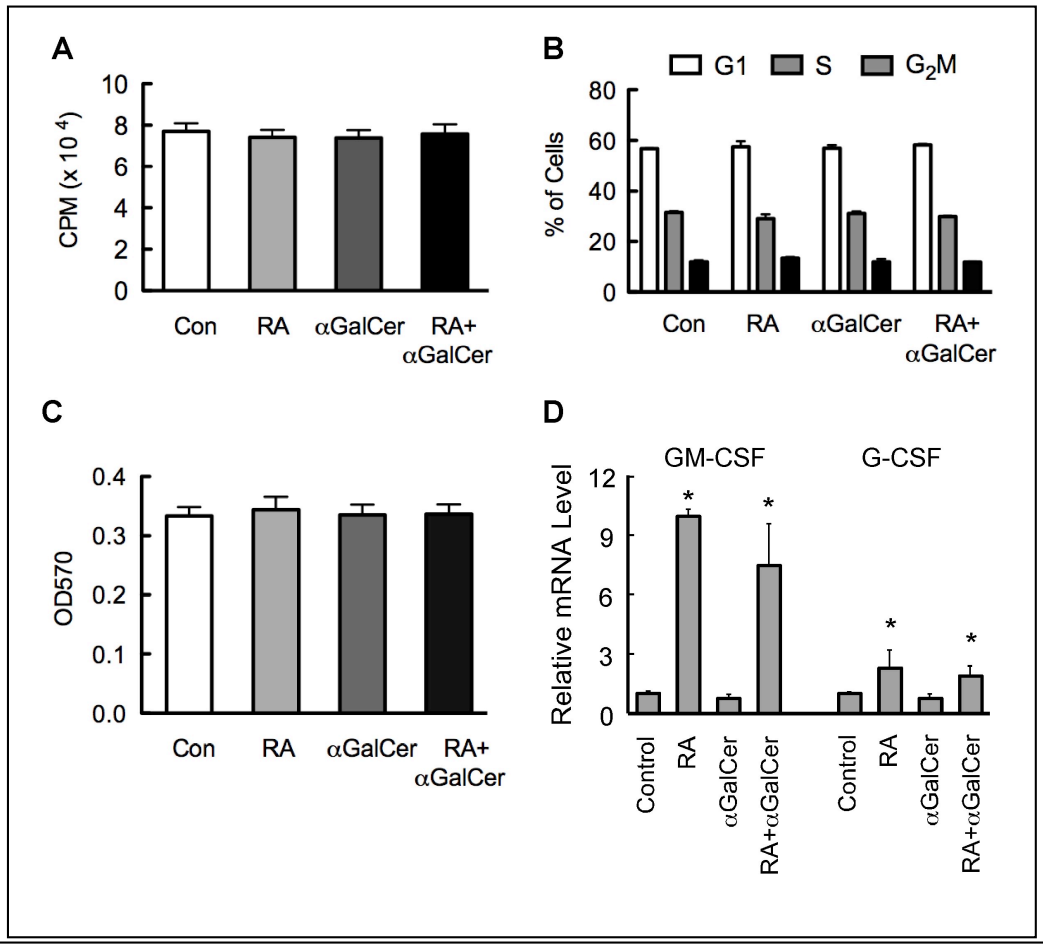

Figure 13. Neither RA nor αGalCer directly affect the growth or motility of 4T1 cells in vitro.

A. 4T1 cells were cultured in the presence and absence of RA (20 nM) and α-GalCer (100 ng/ml) for 24 hours. One μCi/well of ^{3}H-thymidine was added for the final 4 hours and the cell-incorporated ^{3}H was measured using a cell harvester. Data are shown as the means ± SEM of the CPM/well (*n*=6 wells/treatment). None of the groups differed statistically from each other.

B. 4T1 cell cycle distribution was determined by flow cytometry analysis. After culture as in A, the proportion of cells in the G1, S and G2/M phases of the cell cycle were determined using propidium iodide staining and cell cycle analysis with MultiCycle software, n=3 replicates per treatment. None of the groups differed from each other.

C. For the migration assay, cells were cultured under conditions as in A, using standard micro-porous filter plates. The cells that had migrated through the filter pores were stained with crystal violet dye. After washing the filters, the dye was eluted from the filter and determined at its maximum absorption, 570 nm, by spectrophotmetry. Data are shown as the means ± SEM (n=3 wells/treatment). None of the groups differed from each other.

D. The ability of 4T1 cells to produce GM-CSF and G-CSF was tested using a qPCR assay with gene-specific primers. Both RA and the combination of RA and α-GalCer caused a significant increase in GM-CSF and G-CSF expression, which was significant by ANOVA, *P*<0.05 (*) compared to the control group and to α-GalCer alone. This indicates that RA is the major factor increasing these factors in 4T1 cells.

We also surveyed the gene expression of several chemokines that have been related to tumor progression, such as CXCL-1, CCL2, CCL3, CCL4, CCL5 and KLF. Although each gene was expressed at a different abundance in 4T1 cells (Supplementary table 1), none was affected by the presence of RA and α-GalCer, nor were MT1-MMP and MMP3 mRNA (data not shown). In the table, genes with higher values for # of CT (Cycle Threshold) are expressed at lower relative abundance and those with lower values are expressed at higher relative abundance. Overall, in 4T1 cells tested directly, only G-CSF and GM-CSF were responsive to treatment with RA and α-GalCer, as shown in Figure 13 above.

Original task 19. Not initiated. This task was to prepare tissue post-nuclear supernatants, assay protein, run SDS-PAGE gel and do western blot for IRF-1. Explanation: We focused instead on 4T1 tumor cells in the lungs as described above and on MMP expression.

Completed. Original task 20. This task was to repeat tasks 15-19 to increase sample size to n=5-6/group, completing aim 3. We did this. We also added analyses of MMPs and CCL genes, which are reported in task 18 above.

Completed. Milestones 6 and Milestone 7. These were to complete the in vivo studies in aim 3 which tested the potential for RA and α-GalCer, in vivo, to reduce the growth of 4T1 tumors in the lungs, and to explore potential mechanisms, including suppression of production of MMPs by RA and α-GalCer, or both in combination. We completed aim 3 by analyzing the in vivo data and supplementing this with the in vitro studies of 4T1 cells (proliferation, cell cycle phases, migration, and GM-CSF and G-CSF production).

Completed: Original Task 21. This task was to **c**omplete analysis of all data by 2-factor ANOVA. We accomplished the integration of our data, based on studies with both the orthotopic and hematogenous models and supporting studies in cultured cells, including MMP results. We used either 2-way or 1-way ANOVA as appropriate for each design. The results are included in the figures or figure legends shown above.

Completed. Milestone 8. Time line: end of no-cost extension year. We summarized all data and integrated results of studies 1, 2 and 3. Dr. Chen and I gave a poster of our results at the Era of Hope meeting in Orlando in August 2011 (Appendix).

Key Research Accomplishments……..……..

• DC, regardless of treatment, improved the response to 4T1 cells in vivo. Although this is encouraging, we could not demonstrate that treatment of the DC ex vivo with RA or α-GalCer, modified the outcome in vivo. This may be because the loading was not sufficient. Our result does not agree with the work of Toura et al. (5), who described this procedure with α-GalCer as being effective in reducing tumors in a B16 lung tumor model. However, we were encouraged that the DC, whether RA-treated or α-GalCer-loaded or not, as compared to no DC reduced tumor growth in both our orthotopic model and hematogenous model of 4T1 cell breast cancer.

• Our most interesting and potentially important finding was that the combination of RA and α-GalCer, each given to intact mice after tumor injection, was effective in significantly reducing the micrometastatic growth of 4T1 cells in the lungs (Fig. 10). Thus, injection of DC was not essential.

• Mice lacking CD1d did not respond to RA+α-GalCer in vivo, implying that expression of CD1d is necessary for the reduction in lung metastases by RA+αGalCer treatment (Fig. 11).

• The level of expression of MMPs in lung tissue was reduced by RA, while RA plus α-GalCer reduced MMP3 protein in plasma and its production and secretion by 4T1 cells (Fig. 12). This could suggest a possible mechanism for further study.

Inventions, patents and licenses: None

Reportable outcomes

A manuscript was submitted for peer review and has now been published. It is included in the Appendix.

There were no products, prototypes, new animals models or databases generated.

Conclusion

Our research has shown that 4T1 breast tumor cell injection caused tumor formation in the lungs of mice within a short period of time (~12 days). It also resulted in moderate systemic changes in immune system cells which included enrichment of CD11b-positive myeloid cells, of which granulocytes are the dominant cells. Animals treated with RA and exhibited a reduced rate of lung metastasis as demonstrated by histological staining as well in our micrometastasis cell culture assay. A decreased espression level of certain MMPs, especially MMP3, was observe, which could help to explain the mechanism through which RA decreases the spread of 4T1 cells to the lungs.

The basic idea proposed in this IDEA award was that DC treated with RA and α-GalCer may be able to suppress breast tumor growth, or that RA and α-GalCer might be effective when administered directly to mice in vivo, after tumor inoculation. Our study has found some support for an effect of DC, but we did not observe consistent differences due to pretreatment of the DC with RA or α-GalCer. Thus, treatment with autologous DC appears to be promising. As shown in Table 2 (Task 4) we did find that RA increased CD1d expression on isolated cultured murine DC. It may be that the enrichment was not quantitatively large enough for extra binding of α-GalCer. Experiments to further enrich DC and to determine the binding of α-GalCer were outside this project, but we believe that further characterizing the cells that respond to RA with increased CD1d would be an important goal. We notice that these cells were enriched in the "A" gate of slightly larger cells expressing more Ia, and it is possible that sorting DC on cell size and Ia content, using anti-Ia antibodies for detection, would further enrich the DC population with higher CD1d expression. If enough of these enriched DC could be obtained, it would be interesting to try again to use the pulsed DC in vivo in the 4T1 model.

We are not yet convinced that RA-treated, α-GalCer-pulsed DC were completely ineffective, compared to DC alone, as some of the treated mice had smaller tumors. However, overall the variability in the data precluded drawing the conclusion that pretreatment of the DC with RA and α-GalCer ex vivo, before delivery, had a significant effect on 4T1 tumor growth in vivo. The 4T1 breast cancer model is known as an aggressive tumor. Nevertheless, although the tumor grows rapidly, we observed that it remained quite local and encapsulated when it was injected into the mammary gland fat pad. By the time that lung metastases were apparent in our studies, the mammary pad tumors were of such a size that the mice had to be euthanized. We therefore tested a hematogenous model in years 2 and 3, directly injecting the 4T1 cells into the circulation and studying lung metastatases after ~14 days. The hematogenous model proved more tractable, and the reduced number of tumor cells in the lungs of RA+α-GalCer treated mice is quite encouraging.

The finding that MMP3 mRNA is significantly reduced by RA is also encouraging, with MT-MMP1 tending in the same direction. RA could influence metastasis to the lungs by limiting the tumor-induced expression of these genes. α-GalCer did not have the same effect, nor did it interact with RA. Future studies should be conducted to confirm whether RA represses MMP3 expression, determine the mechanism, and whether other MMPs are similarly affected in the lungs or other tissues (liver) of 4T1 cell-injected mice.

Balb/c mice that received the 4T1 cell tumors, either orthotopically or by the hematogenous route, responded immunologically to the tumor cells. This is noteworthy because this is syngeneic model in which direct immunological rejection of the tumor cells are not expected. However the significant increase in spleen weight, and increases in $CD11b^+$ monocytic cells and Gr1+ granulocytes, indicates that a significant innate immune system activation was induced by 4T1 cells. We did not observe differences in NK cell numbers (NK1.1+ cells), which we had hypothesized, but it is possible that the existing NK cells were more active after treatment.

Although we did not observe changes in NK or NKT cell number, the results of our final studies with CD1d-null mice indicate that CD1d is necessary for the reduced tumor growth in the lungs. We did not observe an effect of RA plus α-GalCer in CD1d-null mice. The results are somewhat complicated by the fact that tumor growth was not as great overall in the CD1d-null mice, which may reflect that deletion of this gene has relatively widespread effects on the immune system or other tissues. CD1d-null mice tended to be slightly smaller in size and their overall physiological status is likely to be different from the WT control. Nonetheless, there was no difference between CD1d-null mice without treatment and with RA plus α-GalCer treatment, while the difference due to treatment in the WT controls was significant confirmed our previous study. We thus conclude that for RA plus α-GalCer to be effective in vivo, CD1d is an essential component. Further studies addressing NK and NKT cell function, beyond determining cell number, will be important in the future. There are also other mouse models that lack NKT cells (Jalpha18-null mice) and so it would be possible to further test the importance of NKT cells in other immunological models.

Even though the overall results do not fully confirm our initial hypothesis, they support parts of it. The results have indicated DC can have a potential benefit, and they indicate that RA and α-GalCer administered directly to the tumor-bearing host in vivo can play a modulating role on tumor growth, and particularly on the spread of tumor to the lungs. Since metastasis is the most frequent cause of breast cancer mortality, it is very important to find treatments that can prevent or slow the rate of metastases and tumor growth after metastasis.

Finally, we note that the in vivo treatments with RA and α-GalCer were very well tolerated, with no reduction in body weight and similar body condition in all treatment groups. Thus, RA and α-GalCer could be useful as immunological adjuvants. RA is a potent compound and its clinical application has been limited due to dose-dependent side effects in some clinical studies. Thus determining whether a lower dose of RA would still be effective in our mouse model would be an important future step. There are also analogs of all-*trans*-RA, such as the retinobenzoic acid Am580, that may be more selective and less likely to cause side effects than RA, and therefore it would be very interesting to perform similar experiments using Am580 instead of RA. If it could be shown to work as well as RA does, then it could be considered advantageous for future clinical trials.

Overall, we conclude that this Idea grant, which was reviewed as high risk and novel, has accomplished the majority of its goals. Some pitfalls were not anticipated. However the concept of adjuvant treatments including nutrients and immunological activators to stimulate the immune system to reduce metastases could be relevant to treatment of breast cancer as well as other types of cancer.

Personnel

Key personnel throughout the project
A. Catharine Ross, Ph.D., Principal Investigatory
Qiuyan Chen, M.D., Ph.D., Research Associate

Additional personnel who participated in a limited period
Katherine Restori, B.S., participated in year 3 as a summer graduate student
Amanda E. Wray, M.S., participated in year 3 as a technician (assay support)

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Appendix Cover Page

1. Chen and Ross publication, NUTRITION AND CANCER
2. Supplementary table 1 (Gene primers and cycle threshold levels)
3. ERA OF HOPE abstract, 2008
4. ERA OF HOPE poster, 2011

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All-Trans-Retinoic Acid and the Glycolipid α-Galactosylceramide Combined Reduce Breast Tumor Growth and Lung Metastasis in a 4T1 Murine Breast Tumor Model

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Vitamin A compounds are promising for cancer prevention and reducing risk of recurrence. Herein we have evaluated the combination of all-trans-retinoic acid (RA), a vitamin A metabolite, and alpha-galactosylceramide (αGalCer), a lipid immune activator, in Balb/C mice inoculated with syngeneic 4T1 breast tumor cells on reduction in breast tumor growth and lung metastasis. In Balb/c inoculated with the syngenic 4T1 primary tumor, and administered dendritic cells treated with RA + αGalCer, the size of the primary tumor and the number of lung metastatic foci were reduced. When 4T1 cells were introduced into the circulation as a model of hematogenous spread of tumor cells and RA and αCalCer were administered directly to mice without dendritic cells, lung metastatic foci were reduced 70% ($P < 0.05$), whereas each agent alone resulted in an intermediate decrease. Concomitantly, the expression of matrix metalloproteinases (MMP), membrane type-1 (MT1)-MMP and MMP3, were reduced by RA + αGalCer in lung. MMP3 protein was also reduced in plasma and culture supernatants from RA + αGalCer-treated 4T1 cells. Together, our results provide new evidence that a nutritional-immunological combination of RA + αGalCer may be promising for preventing or slowing the growth of metastatic foci, and suggest reduced MMP production as a possible mechanism.

INTRODUCTION

Breast cancer is the most commonly diagnosed cancer and is the leading cause of cancer-related deaths in women (1). Many bioactive compounds acquired from the diet or natural sources have received increasing attention for the development of novel agents to prevent the occurrence and progression of cancer. Among such candidates, some agents not only target the cancer cells, but also induce the immune system to greater surveillance and elimination of transformed cells. A combination of treatments may produce additive or synergistic effects, and thus strategies using multiple nutritional or nutrient–drug combinations may prove fruitful for preventing and/or reducing the spread of cancer cells.

All-trans-retinoic acid (RA), an active metabolite of vitamin A, is well-known for its regulatory roles in cell growth and differentiation (2). Numerous animal and human studies have reported that RA is important in maintaining an efficient immune response (3, 4), and a few clinical studies have also suggested that RA–vitamin A supplementation may be beneficial in immune disorders and cancer (5–8).

α-Galactosylceramide (αGalCer) is a glycolipid that has become the prototype of glycolipid antigens used in experimental studies and clinical trials. αGalCer has entered Phase I/II clinical trials (9, 10). αGalCer is known to stimulate a subset of T cells bearing characteristics of natural killer (NK) cells, referred to as invariant natural killer T (iNKT) cells, through the presentation of αGalCer by a Major Histocompatibility Complex (MHC)-like receptor CD1d (11, 12). The dependence of the antitumor effect of αGalCer on iNKT cells was demonstrated in studies that showed no protective antitumor activity in $J\alpha18^{-/-}$ mice, which lack iNKT cells (13–16), whereas iNKT mice, which have only iNKT cells and no conventional T cells and NK cells, showed antitumor activity equivalent to wild-type (WT) mice (17). As numerous studies have provided evidence that αGalCer plays a role in the suppression of tumor growth and metastasis, it has become an interesting component of immune therapy (10, 18–22). Furthermore, injection of αGalCer-loaded dendritic cells (DCs) has elicited promising effects in inhibition of tumor growth and metastasis, both experimentally and in clinical trials (23–25), suggesting that the activation of DCs may play an important role the downstream immune responses.

Based on previous observations that RA can markedly increase CD1d gene expression (26, 27), we hypothesized that RA might be effective for enhancing the antitumor activity of the CD1d ligand αGalCer. In the current study, we have used as our model the 4T1 tumor, originally derived from a spontaneously arising Balb/c mammary tumor (28), which has been well

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characterized and shown to have several advantages, including that the tumor cells are easily transplanted into the mammary gland and multiple distant metastatic foci develop spontaneously from the primary tumor via hematogenous or lymphatic routes, similar to that of human breast cancer. In addition, 4T1 cells are resistant to growth inhibition by 6-thioguanine, which makes it possible to determine the presence of metastatic cells by ex vivo culturing lung homogenates in the presence of this compound, even if the tumor foci are too small to be counted microscopically (28). Using different approaches, we have tested the combination of RA and αGalCer for their ability to reduced 4T1 breast tumor growth and lung metastasis.

MATERIALS AND METHODS

Cell Culture and Reagents

4T1 cells were kindly provided by Dr. Danny Welch (University of Kansas Cancer Center) and maintained in RPMI 1640 supplemented with 10% fetal calf serum and 2×10^{-5} M ß-mercaptoethanol to facilitate the cell growth in vitro (Invitrogen, Grand Island, NY).

Splenic DCs were isolated from control Balb/c mice using a dendritic cell isolation kit according to the manufacturer's instruction (Stemcell Technology, Vancouver, Canada); cell purity was up to 87%. DCs were treated ex vivo with RA (20 nM, from Sigma-Aldrich, St. Louis, MO) for 16 h and then treated again with RA, plus TNFα (5 ng/ml, R&D Systems, Minneapolis, MN), an agent used to promote DC maturation (29), and αGalCer (100 nM, Enzo Life Sciences, Farmingdale, NY) for 2 h, then washed twice with PBS (Invitrogen). We immediately injected 1×10^5 cells per mouse via the retro-orbital sinus.

Animals and Experimental Design

Protocols were approved by the Institutional Animal Use and Care Committee of Pennsylvania State University. Eight-wk-old female Balb/c mice (Charles River, Wilmington, MA) were housed in plastic air-filtered cages with free access to water and standard chew diet.

For the orthotopic model, mice were inoculated with 2×10^4 4T1 cells in 100 μl PBS directly into the inguinal mammary fat pad. RA and αGalCer-pulsed DC were injected retro-orbitally on the same days. Mice were closely monitored and once the tumors reached between 0.5–1.0 cm, about 28 days after 4T1 cell inoculation, all of the mice were sequentially euthanized with carbon dioxide, tumor nodules were dissected and weighed, lungs were subjected to histology and micrometastasis assay, and spleens were collected, weighed, and used for flow cytometry.

In the hematogenous model, 2×10^4 4T1 cells in 100 μl PBS were injected directly into the circulation to model the spread of tumor cells (30). Twenty-four h later, mice were randomized to receive control (vehicle as Canola oil), RA, αGalCer, or both. All-trans-RA, 37.5 μg per mouse in Canola oil, was given orally once a day for 10 days. αGalCer, 2 μg per mouse, was given subcutaneously every other day.

Micrometastasis Assay

As adapted from a previous report (31), lung tissues (25 mg from each individual sample) were minced and digested with 10 mg/ml of collagenase type IV and 10 U/ml of elastase (Worthington Biochemical Corporation, Lakewood, NJ), for 30 min, and a single-cell suspension was prepared by passing the sample through a nylon mesh. Cells were then washed and serially diluted in culture medium containing 60 μM of 6-thioguanine (Sigma-Aldrich, St. Louis, MO) (28), and cultured for 7 to 10 days in 6-well plates. Colonies were washed with PBS, fixed with methanol, and then stained with 0.1% crystal violet to facilitate the counting of colonies. After counting, the dye was dissolved in 10% acetic acid and the absorbance at 570 nm was measured as an integrated measure of colony size and number.

Flow Cytometric Analysis

Splenocytes were prepared by Histopaque (Sigma-Aldrich, St. Louis, MO) centrifugation and analyzed for cell populations by staining with fluorescently tagged monoclonal antibodies: CD19-PEcy7, CD3-FITC, CD4-PerCP, CD8-APC, NK1.1-PEcy7, CD11c-PEcy7, F4/80-APC, Gr-1-FITC, and CD11b-PE, all from BD Biosciences (San Diego, CA). Five $\times 10^5$ cells were incubated with 0.1 μg of respective antibody or combination for 1 hour at room temperature (32). The channel selection was optimal for each fluorochrome measured using an Accuri C6 flow cytometer (Accuri, BD Biosciences, San Jose, CA). Appropriate isotype controls were used as negative controls and single-stained samples were used to set the proper compensation.

Cell Proliferation Assay

4T1 cells were plated in 96-well plates, 2×10^4 cells/well/200 μl, and treated with or without RA (20 nM) or αGalCer (100 ng/ml) for 24 h. [^{3}H]-Thymidine (PerkinElmer, Inc, San Jose, CA) was added for the last 4 h (0.1 μCi/50 μl/well) for determination of [^{3}H]-thymidine incorporation as a measure of cell proliferation (32).

Cell Cycle Analysis

4T1 cells were plated in 48-well plates, 1×10^5 cells/well/0.5 ml, and treated as above with or without RA, or αGalCer, for 24 h and then harvested and subjected to propidium iodide staining and cell cycle analysis (32).

Motility Assay

Five $\times 10^4$ 4T1 cells were plated in serum-free RPMI 1640 medium in the top chamber of a transwell plate (Corning, NY), separated from the bottom by a polycarbonate membrane with 8-μm pores. Medium with 10% FBS containing RA (20 nM), αGalCer (100 ng/ml), or both was placed in the bottom chamber. After a 24-h culture, nonmigrated 4T1 cells were removed with a cotton swab from the upper surface of the membrane. Cells that migrated through and adhered to the lower surface were stained with 0.1% crystal violet and rinsed with deionized water.

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The dye was eluted with 10% acetic acid and quantified by spectrophotometer as described above.

Quantitative Real-Time PCR (qPCR)

Lung tissues, or 4T1 cells cultured for 24 h, as above, were used to prepare total RNA (Qiagen, Valencia, CA). A SYBR green master mixture (Biorad, Hercules, CA) was used for the quantification of PCR products, and quantified relative to amplification of 18S RNA (27). Primer sequences are listed in Table 1.

ELISA of Matrix Metalloproteinases (MMP) 3

MMP3 protein was measured in mouse plasma samples (1:20 dilution) and 4T1 cell culture medium (without dilution) using an ELISA kit for mouse MMP3 (Abcam, Cambridge, MA) according to manufacturer's instruction.

Statistic Analysis

Results are expressed as the mean ± SEM. Data was analyzed by *t*-test for 2 groups or analysis of variance followed by Tukey's post-hoc test, using Prism software (Graphpad Software, La Jolla, CA), unless specified in legends. $P < 0.05$ was considered statistically significant.

RESULTS

RA and αGalCer-Treated Syngeneic DC Reduced the 4T1 Cell Growth and Metastasis in a Mouse Orthotopic Breast Tumor Model

It has been reported that αGalCer-loaded DCs can inhibit the growth of several different tumors (23–25, 33). We hypothesized that αGalCer may have a similar effect on inhibiting the growth of breast tumor cells, and RA may augment the effect

TABLE 1
Primers used in the qPCR analysis, and the gene expression level in 4T1 cells determined as threshold cycle (CT) number

Name	Primer sequence	# of CT
G-CSF (granulocyte colony stimulating factor)	F: 5-ATGGCTCAACTTTCTGCCCA R: 5-ACATCCAGCTGAAGCAAGTCCA	16.70 ± 0.08
M-CSF (macrophage colony stimulating factor)	F: 5-ATAAAGGCACGCAGCCCAAA R: 5-AAACCTGGCTTGCTTGGCTA	21.67 ± 0.13
GM-CSF (granulocyte macrophage colony stimulating factor)	F: 5-TCCGGAAACGGACTGTGAAACA R: 5-TGCCACATCTCTTGGTCCCTTT	19.88 ± 0.31
CXCL1 (C-X-C motif ligand 1)	F: 5-AACGCTGGCTTCTGACAACA R: 5-AAACACAGCCTCCCACACAT	19.20 ± 0.07
CCL2 (C-C motif ligand 2)	F: 5-AGGTGTCCCAAAGAAGCTGT R: 5-TGCTTGAGGTGGTTGTGGAA	20.22 ± 0.17
CCL3 (C-C motif ligand 3)	F: 5-TGAAACCAGCAGCCTTTGCT R: 5-ATGCAGGTGGCAGGAATGTT	25.43 ± 0.16
CCL4 (C-C motif ligand 4)	F: 5-AACACCATGAAGCTCTGCGT R: 5-AGCTGCTCAGTTCAACTCCA	26.70 ± 0.11
CCL5 (C-C motif ligand 5)	F: 5-TGCCCACGTCAAGGAGTATT R: 5-CAGGACCGGAGTGGGAGT	18.93 ± 0.05
KLF13 (Kruppel-like factor 13)	F: 5-TGGCAGGAGTGCAACAAGAA R: 5-CTCAAGTTCGCTCAGCTTTCCT	19.20 ± 0.12
MMP2 (matrix metalloproteinase 2)	F: 5-GACATCAAGGGGATCCAGGAGCTC R: 5-TCAGCAGCCCAGCCAGTCTGATTTG	UD
MMP3 (matrix metalloproteinase 3)	F: 5-GGAACCTGAGACATCACCAATGTGC R: 5-AGCTATTGCTCTTCAATATGTGGGT	20.22 ± 0.08
MMP9 (matrix metalloproteinase 9)	F: 5-CAGCCCCTGCTCCTGGCTCTCCTG R: 5-ACTCGTCGTCGTCGAAATGGGCAT	UD
MT1-MMP (membrane –type 1 matrix metalloproteinase)	F: 5-GGAACCCGGGTACGCCAACCACATTA R: 5-TCAGACCTTGTCCAGCAGCGAACGC	19.32 ± 0.12
HPRT (hypoxanthine-guanine phosphoribosyltransferase)	F: 5-AAGGACCTCTCGAAGTGTTGGATA R: 5-CATTTAAAAGGAACTGTTGACAACG	25.27 ± 0.21
18S (18S ribosomal RNA)	F: 5-AATGGTGCTACCGGTCATTC R: 5-ACCTCTCTTACCCGCTCTCC	15.31 ± 0.37

CT was set at the same level for every gene. HPRT and 18S served as reference genes. UD: Undefined. UD in 4T1 cells, whereas the mRNA level in lung was not changed after treatment.

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of αGalCer-stimulated DCs. In the present study, isolated DC were treated ex vivo with a physiological concentration of RA (20 nM) for 16 h and then loaded with αGalCer (100 ng/ml) for 2 h. The DCs were intravenously injected into Balb/c mice at the same time that 4T1 cells were inoculated into the inguinal mammary gland.

Tumors were palpable at the injection sites 2 wk after injection. By 4 wk, tumors in some mice had grown to ~1 cm and all mice were then euthanized. The 4T1 cells formed a solid tumor mass near the injection site, which often consisted of one or more encapsulated well-defined nodules, without apparent local invasion. Whereas untreated DCs or DCs pretreated with RA or αGalCer alone did not result in a significant change (data not shown), mice treated with DC pretreated with RA + αGalCer had a tendency to bear smaller primary tumor nodules, as shown in Fig. 1A ($P = 0.055$).

To test whether injection of DCs also affects the long-distance metastasis of tumors, we processed the lung and performed a colony formation assay. Mice treated with RA + αGalCer-treated DCs tended to have fewer metastatic foci ($P = 0.051$, Fig. 1B). Representative histology images (Fig. 1C), showed smaller and fewer metastatic foci, and more normal parenchyma tissue, in the lungs of DC-treated mice than in mice that did not receive DC.

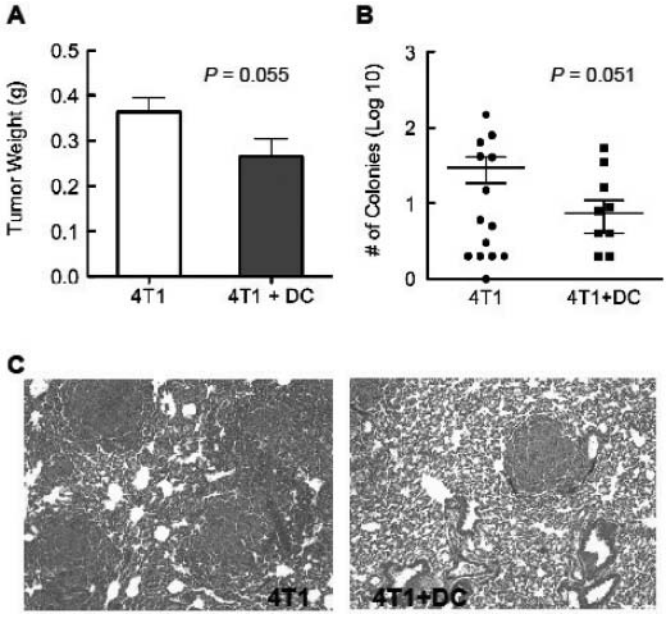

FIG. 1. Treatment of Balb/c mice with all-trans-retinoic acid (RA) and alpha-galactosylceramide (αGalCer)-treated dendritic cells decreased the primary tumor formation and lung metastasis rate in an orthotopic 4T1 breast tumor model. A: Tumor weight. B: Metastasis colony number in the lung. Data were pooled from 3 independent experiments with $n = 4$ to 6 mice/group in each experiment results of *t*-test are shown. C: Representative images (hematoxylin and eosin staining) of lung tissue histology.

4T1 Cell Inoculation Altered Spleen Size and Cell Population

We also observed that the spleen was markedly enlarged in tumor-bearing mice compared to mice without 4T1 cell injection (Fig. 2A), whereas spleen weight was decreased in mice treated with DCs. The weight of the spleen correlated well with the weight of the primary tumor (Fig. 2B, $P < 0.0001$), suggesting a chronic response of the immune system to the growth of the tumor cells (34, 35).

Flow cytometric analysis revealed that the spleen enlargement was due to an alteration of cellular components. $CD11b^+$ cells were dramatically increased (Fig. 2C), mainly because of an increase in $Gr\text{-}1^+$ granulocytes, whereas, conversely, T and B lymphocytes, which normally comprise the major cell populations of the spleen and contribute over 80% of the total cells,

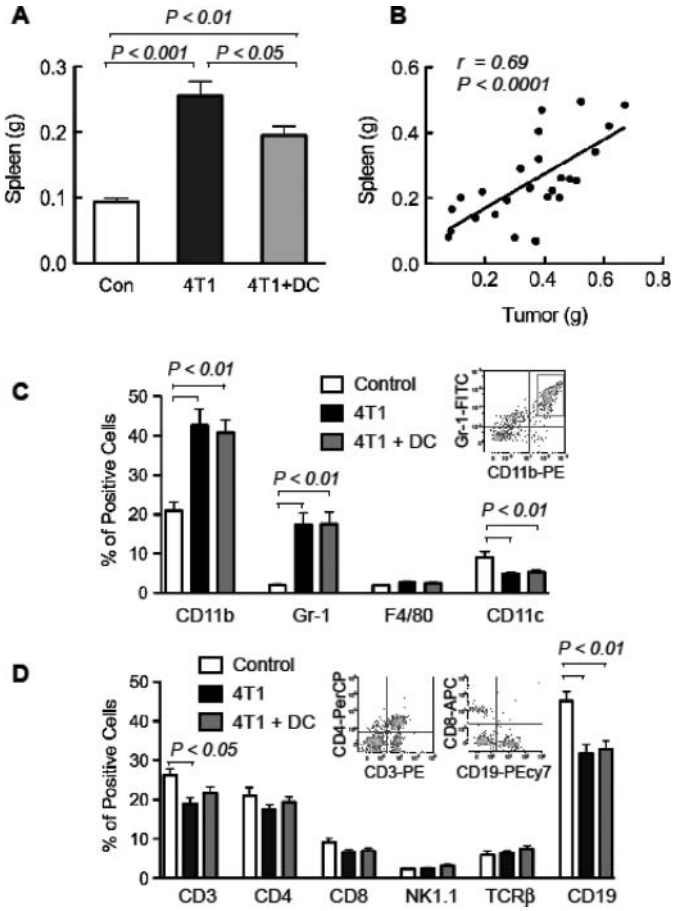

FIG. 2. 4T1 cell inoculation in Balb/c mice resulted in spleen enlargement and cell population changes. A: Spleen weight. B: Correlation analysis of spleen weight and tumor nodules weight, $P < 0.0001$. C: Staining for $CD11b^+$ cell population, most of which were $Gr\text{-}1^+$ cells in 4T1-inoculated mice. A representative scatter plot showing the population measured is shown in the upper right quadrant of the boxed panel. D: Splenic lymphocytes were determined in Balb/c mice after 4T1 inoculation. Representative scatter plots are inserted to show the CD3, CD4, CD8, and CD19 staining and gating. Data were pooled from 3 independent experiments, as in Fig. 1.

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were decreased in every subpopulation, especially the B cell subset (Fig. 2D). The percentage of $CD11c^+$ dendritic cells was also reduced in 4T1-bearing mice.

RA and αGalCer Decreased 4T1 Cell Metastasis to the Lungs After Intravenous Injection of 4T1 Cells

We next investigated whether RA and αGalCer alter tumor cell metastasis in mice without treatment with DCs. In Experiment 2, mice were inoculated with 4T1 cells directly into the blood stream as a model of the hematogenous spread of tumor cells, and then treated in vivo with multiple doses of RA and/or αGalCer. After 14 days, 4T1 cells in lung tissue were evaluated. With this procedure, the weight of the spleen did not increase nearly as greatly as in the previous experiment (data not shown), which likely reflects the shorter time of this experiment because the number of tumor cell inoculated was the same for both this and our first experiment. However, spleen cell populations were still altered, similar to Experiment 1, with decreased lymphocytes and increased $CD11b^+$ cells, most of which were $Gr\text{-}1^+$ cells (Fig. 3A and 3B). Treatment with RA and/or αGalCer did not alter the population changes that were caused by the 4T1 tumor cells.

Either RA or αGalCer alone reduced the rate of lung metastasis nonsignficantly, determined both by counting the lung tissue colony number and staining the colonies. However, RA + αGalCer combined decreased lung metastasis significantly ($P < 0.05$, Fig. 3C and 3D).

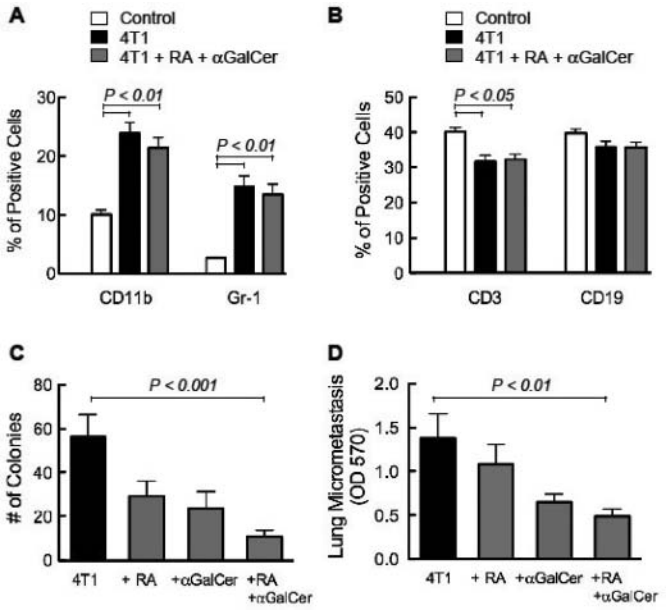

FIG. 3. All-trans-retinoic acid (RA) and and alpha-galactosylceramide (αGalCer) decreased lung metastasis in a hematogenous model of 4T1 cell inoculation. Spleen myeloid cells (A) and lymphocytes (B) were determined by flow cytometry. C: Micrometastasis was determined by counting the number of colonies on each culture plate. D: Micrometastasis was determined by measuring the absorbance at 570 nm, after crystal violet staining, which represents size and number of colonies. Data were combined from 3 independent experiments with $n = 5$ or 6 mice in each experiment.

CD1d Deficiency Attenuated the Effect of RA and αGalCer In Vivo

To further study the mechanisms of RA and αGalCer in the prevention of tumor metastasis, we employed CD1d null mice to determine whether the effect of RA + αGalCer in reducing tumor metastatic growth is CD1d dependent. 4T1 cells were inoculated intravenously in both WT and CD1d-null strains and the lung metastasis rate was determined 14 days later, as in the previous study. After treatment with RA + αGalCer, tumor metastases were lower in WT mice, but not in CD1d-null mice (Fig. 4A).

We then determined if the splenic cellular response observed previously was altered by CD1d deficiency. Both WT and CD1d-null mice exhibited a similar marked increase in $CD11b^+$ cells and, to a lesser degree, a decrease in lymphocytes, especially B cells. However, those CD1d-null mice treated with RA + αGalCer had higher percentages of CD11b/$Gr\text{-}1^+$ cells and lower B cells, suggesting that CD1d is also important for limiting the magnitude of CD1b/$Gr\text{-}1^+$ cell expansion (Fig. 4B and 4C).

RA and αGalCer Decreased the Level of Expression of MMPs in Lung Tissues

It is known that MMPs, a family of zinc-dependent endopeptidases capable of degrading the extracellular matrix, play important roles in tumor invasion and metastasis (36, 37). We first screened the gene expression levels of several MMPs in lung tissue by quantitative real-time RT-PCR (Table 1). Among the several MMPs reported in the literature, the inoculation of mice with 4T1 cells greatly increased the gene expression levels of membrane-type 1 MMP (MT1-MMP) and MMP3 (Fig. 5A). We then assessed MT1-MMP and MMP3 mRNA levels in the lungs of mice with and without treatment with RA and αGalCer. Mice treated with RA and αGalCer showed significantly decreased levels of both MT1-MMP and MMP3 mRNA in lung. To further examine MMP expression, MMP3 protein was measured in plasma. As shown in Fig. 5B, the level of MMP3 in plasma was marginally increased in 4T1 tumor-bearing mice, although it was reduced by treatment with RA + αGalCer. MMP3 protein in 4T1 cell culture supernatant was also lower in mice treated with either RA or αGalCer alone, as well as both in combination (Fig. 5C). These data suggest that inhibition of MMP production by 4T1 tumors may be one of the mechanisms by which RA and αGalCer can reduce the rate of lung tumor metastatic growth.

RA and αGalCer Did Not Alter 4T1 Cell Growth or Migration In Vitro

To determine if RA and αGalCer have a direct effect on 4T1 cells, several properties were tested. In a 24-h assay, cell proliferation did not differ between each treatment and control cells (Fig. 6A). Treatment also did not affect the distribution of

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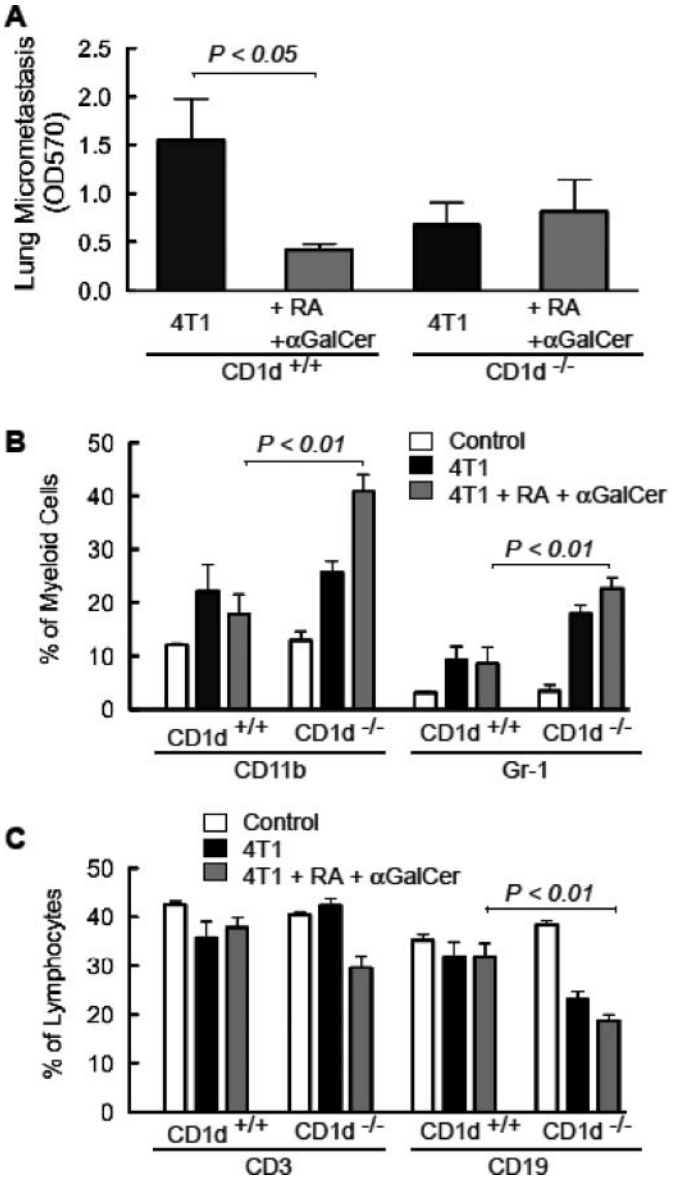

FIG. 4. 4T1 tumor metastasis in wild type and CD1d$^{-/-}$ mice. A: Lung metastasis rate represented by the absorbance at 570 nm. B: Splenic myeloid cell population. C: Lymphocyte staining. The data shown were combined from 2 independent experiments with $n = 5$ or 6 mice in each experiment.

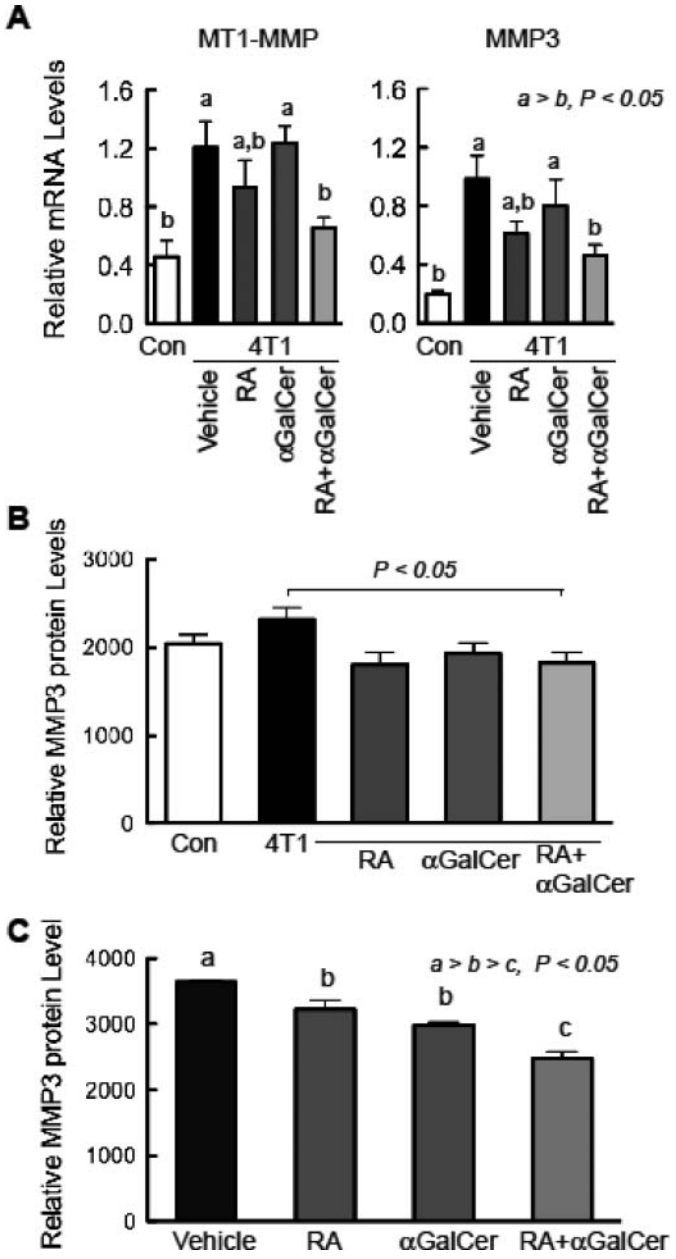

FIG. 5. Matrix metalloproteinase levels in vivo and in cultured 4T1 cells. A: Membrane-type 1 matrix metalloproteinases (MT1-MMP) and MMP3 mRNA levels in lung tissues. B: MMP3 protein in plasma of the animals described in Fig. 3, determined by ELISA. C: MMP3 protein level determined by ELISA in the culture supernatant of 4T1 cells cultured in the presence and absence of all-trans-retinoic acid (RA; 20 nM) and alpha-galactosylceramide (αGalCer; 100 ng/ml) for 24 hr. The data shown represents 2 independent experiments each conducted in triplicate.

cell cycle stages (Fig. 6B). Cell migration in response to RA and αGalCer, tested using a 16-h transwell culture assay, also did not show difference between each treatment and control cells (Fig. 6C).

RT-PCR analysis was used to determine the expression levels of granulocyte colony stimulating factor (G-CSF), macrophage colony stimulating factor (M-CSF), and granulocyte macrophage colony stimulating factor (GM-CSF) in 4T1 cells after RA and/or αGalCer treatment in vitro. RA significantly upregulated GM-CSF gene expression of the 4T1 cells (Fig. 6D); however, αGalCer had no effect on the cytokine expression. G-CSF and M-CSF expression were not affected by either RA or αGalCer (data not shown).

We also surveyed the gene expression of several chemokines that have been related to tumor progression, such as C-X-C motif ligand 1 (CXCL1), C-C motif ligand 2 (CCL2), C-C motif ligand 3 (CCL3), C-C motif ligand 4 (CCL4), C-C motif

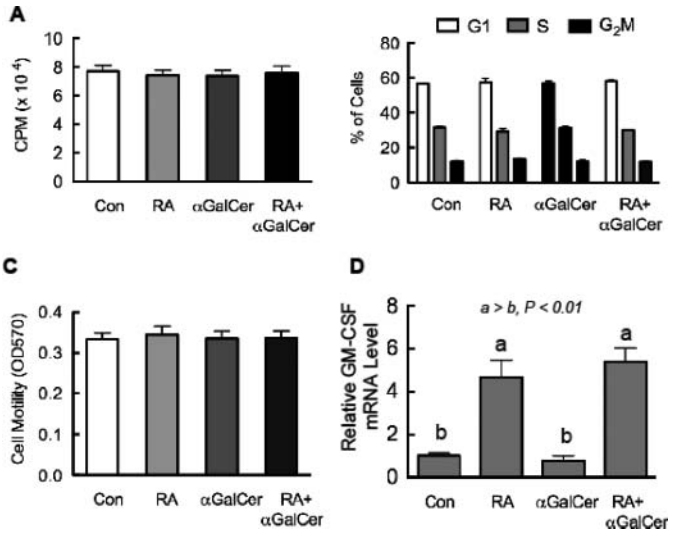

FIG. 6. Effects of all-trans-retinoic acid (RA) and and alpha-galactosylceramide (αGalCer) on 4T1 cell growth, cell motility, and gene expression in vitro. 4T1 cells were cultured as in Fig. 5C. A: Cell proliferation was measured by [^{3}H]-thymidine incorporation. B: 4T1 cell cycle distribution was determined by flow cytometric analysis. C: 4T1 cell motility was measured in a transwell assay and cells that migrated cells were quantified as the absorbance at 570 nm after staining with crystal violet. D: The level of GM-CSF mRNA in 4T1 cells was measured by q-PCR. The data shown represents 2 independent experiments each conducted in triplicate.

ligand 5 (CCL5), and Kruppel-like factor (KLF). Although each gene was expressed at a different abundance (Table 1), none was affected by the presence of RA and αGalCer, nor were MT1-MMP and MMP3 mRNA (data not shown).

DISCUSSION

In the current study, we used a 4T1 mammary carcinoma model to investigate the potential preventive effects of RA and αGalCer on breast cancer growth and metastasis. In the first model in which 4T1 cells were injected into the mammary fat pads of the mice, the administration of syngeneic DCs pulsed with RA + αGalCer tended to suppress the solid tumor growth, although the reduction was only marginally statistically significant. Because neither RA nor αGalCer had observable effects on 4T1 cell growth in vitro, the reduced tumor growth and lung metastasis is likely to be due to immune stimulation, or other effects mediated by the response of the animals. Because the spread of tumors to the lungs seemed to have been prevented or tumor growth reduced, we then tested the effect of RA and αGalCer when given directly to mice inoculated with 4T1 cells intravenously. In this way, the number of cells introduced could be kept equal among groups, and the effect of RA and αGalCer on lung tumor growth in vivo could be studied directly. This study showed that RA + αGalCer is an effective combination in preventing or reducing the development of micrometastasis in the lungs. CD1d, a receptor for αGalCer (38), was shown to be required, as the effect of RA + αGalCer compared to control treatment that was observed in WT mice was not observed in CD1d-null mice.

It is known that both RA and αGalCer can act on the immune system. RA has a broad range of activities in the regulation of immune competent cells, such as activation of NK cells, inducing differentiation of monocytes, B and T cells. The effects of αGalCer are believed to be mainly due to the activation of NKT cells that can play a crucial role in antitumor responses (12, 33, 39). Moreover, the ability of RA to regulate CD1d gene expression on antigen-presenting cells (26, 27) may be another factor in our results, where the combination of RA + αGalCer was more effective than either agent alone in preventing the growth of lung metastases.

It is reported that the growth of 4T1 tumor can cause a leukemoid reaction, apparent as granulocytosis in which Gr-1$^+$ cells are greatly increased in peripheral blood and spleen, accompanied by granulopoiesis in the bone marrow (40, 41). Moreover, the dim staining of the Gr-1 marker suggested most of the increased number of Gr-1$^+$ cells were immature granulocytes (41). We observed a similar reaction in both of the models we tested. In the orthotopic model (experiment 1), which lasted 28 days, the spleen was significantly enlarged, with a significant increase in Gr-1$^+$ cells. In the intravenous metastasis model (Experiment 2), which lasted 14 days, the weight of the spleen was not dramatically increased; however, the Gr-1$^+$ cells were still increased to a significantly higher level. In both situations, the lymphocyte population was reduced in percentage although the total number of cells was not decreased (data not shown). Because myeloid cells play complex roles in tumor growth and metastasis (42), and the leukemoid reaction with granulocytosis is not a specific phenomenon of the 4T1 tumor, the significance of increased Gr-1$^+$ cell is not clear at the present time. It is reported that the CD11b$^+$Gr-1$_{dim}$ immature granulocyte population is part of the myeloid suppressor cells that can inhibit T cell-mediated tumor immunity (43, 44). However, the Gr-1$^+$ population in our study was mainly CD11b$^+$Gr-1$_{bright}$ cells. As RA is known to induce the differentiation of monocytic cells (32, 45), and αGalCer has a mitogenic effect on the activation of immune cells (46), including the myeloid lineage, future studies are needed to characterize the functional activity of the enriched CD11b$^+$Gr-1$_{bright}$ granulocytes. Our data showed that treatment of 4T1 cells with RA markedly increases the level of gene expression of GM-CSF. However, whereas GM-CSF can promote granulocytosis, we did not observe that RA, in our animal models, further increased spleen size, or affected the Gr-1$^+$ cell population. Thus, the granulocytosis appears to be the consequence of the 4T1 tumor growth in the body. It is reported that the granulocytosis becomes more dramatic with time of tumor residence (41), further suggesting that it is the growth of the tumor cells that causes the change of immune cell populations. Therefore, treatments that reduce tumor growth may be predicted to reduce granulocytosis as well.

Another area that has attracted much attention in cancer prevention concerns MMPs, which, through their ability to

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degrade extracellular matrix, perform a critical function in processes such as tumor angiogenesis, metastasis, and the release of growth factors and cytokines from the extracellular matrix. The circulating levels of MMPs are currently used as prognostic indicators of metastasis (47, 48). Among several MMPs in the gene family, we found MT1-MMP and MMP3 to be elevated in lung tissue after 4T1 tumor growth. RA reduced the expression of both genes. As determined by ELISA, MMP3 protein in plasma was slightly increased after 4T1 cell inoculation and slightly but significantly reduced after treatment with RA + αGalCer. A direct effect on the tumor cells was evident for MMP3, indicated by reduced MMP3 protein produced and secreted by 4T1 cells treated with RA and/or αGalCer. Although the mechanism of the regulation of MMP expression is not clear at the present time, these data suggest an interesting new potential way through which RA and αGalCer may regulate the tumor invasive characteristics by altering the MMP levels.

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ACKNOWLEDGMENT

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Table S1. Primer sequences used for qPCR and the expression level (Cycle Threshold (CT) of each gene

Name	**Primer sequence**	**# of CT** (4T1 cells)
G-CSF	F: 5-ATGGCTCAACTTTCTGCCCA R: 5-ACATCCAGCTGAAGCAAGTCCA	16.70 ± 0.08
M-CSF	F: 5-ATAAAGGCACGCAGCCCAAA R: 5-AAACCTGGCTTGCTTGGCTA	21.67 ± 0.13
GM-CSF	F: 5-TCCGGAAACGGACTGTGAAACA R: 5-TGCCACATCTCTTGGTCCCTTT	19.88 ± 0.31
CXCL1	F: 5-AACGCTGGCTTCTGACAACA R: 5-AAACACAGCCTCCCACACAT	19.20 ± 0.07
CCL2	F: 5-AGGTGTCCCAAAGAAGCTGT R: 5-TGCTTGAGGTGGTTGTGGAA	20.22 ± 0.17
CCL3	F: 5-TGAAACCAGCAGCCTTTGCT R: 5-ATGCAGGTGGCAGGAATGTT	25.43 ± 0.16
CCL4	F: 5-AACACCATGAAGCTCTGCGT R: 5-AGCTGCTCAGTTCAACTCCA	26.70 ± 0.11
CCL5	F: 5-TGCCCACGTCAAGGAGTATT R: 5-CAGGACCGGAGTGGGAGT	18.93 ± 0.05
KLF13	F: 5-TGGCAGGAGTGCAACAAGAA R: 5-CTCAAGTTCGCTCAGCTTTCCT	19.20 ± 0.12
MMP2	F: 5-GACATCAAGGGGATCCAGGAGCTC R: 5-TCAGCAGCCCAGCCAGTCTGATTTG	UD*
MMP3	F: 5-GGAACCTGAGACATCACCAATGTGC R: 5-AGCTATTGCTCTTCAATATGTGGGT	20.22 ± 0.08
MMP9	F: 5-CAGCCCCTGCTCCTGGCTCTCCTG R: 5-ACTCGTCGTCGTCGAAATGGGCAT	UD*
MT1-MMP	F: 5-GGAACCCGGGTACGCCAACCACATTA R: 5-TCAGACCTTGTCCAGCAGCGAACGC	19.32 ± 0.12
HPRT	F: 5-AAGGACCTCTCGAAGTGTTGGATA R: 5-CATTTAAAAGGAACTGTTGACAACG	25.27 ± 0.21
18S	F: 5-AATGGTGCTACCGGTCATTC R: 5-ACCTCTCTTACCCGCTCTCC	15.31 ± 0.37

UD*: Undefined in 4T1 cells, whereas the mRNA level in lung was not changed after treatment.

ERA OF HOPE ABSTRACT, 2008

Retinoic acid and alpha-galactosylceramide, a ligand for CD1d on antigen-presenting cells, differentially regulate the production of immunoregulatory cytokines by cultured dendritic cells and splenocytes

Background and objectives: Activation of the natural immune system is promising as a way to inhibit tumor growth. Dendritic cells (DC), natural killer (NK) cells, and a specialized subset of T cells known as iNKT cells are among the cell types likely to inhibit tumor growth in vivo. Based on previous results, we postulated that a combination of retinoic acid (RA), an agent that often inhibits cell growth and induces cell differentiation, and which can induce the expression of CD1d by antigen-presenting cells, may, in the presence of α-galactosyl-ceramide (αGalCer) which binds to CD1d, augment the activation of iNKT cells. In animals, αGalCer has shown encouraging results against several types of cancer, but to our knowledge it has not been tested in combination with RA or agents such as poly-I:C, a strong inducer of interferons (IFN). We thus have proposed that a triple nutritional-immunological combination of RA, αGalCer and poly-I:C could be effective for breast cancer immunotherapy, based on the ability of: 1) RA to induce CD1d, 2) αGalCer to bind to CD1d on antigen-presenting cells (dendritic cells, DC, or macrophages) and activate iNKT cells, and 3) poly-I:C to stimulate the production of cytokines that activate NK cells. Our research goal is to test whether these agents may synergistically inhibit breast tumor growth in mice. In the first 6-months of our award, we have examined whether RA together with αGalCer regulates the proliferation of DC and splenocytes in culture, and their ability to produce immunoregulatory cytokines.

Methods: DC were prepared from bone marrow of the femur and tibia of adult (> 8 wk) female Balb/c or C57BL/6 mice. GM-CSF was added to the cultures in complete media every 3 days for total of 9 days to induce DC growth. On day 9, TNFα (5 ng/ml) was added to induce DC differentiation. Splenic mononuclear cells were treated with RA ± αGalCer for 24 h. To monitor NKT cell proliferation, differentiated DC or isolated splenocytes in 96-well plates were treated with RA (20 nM) or αGalCer (100 nM) for 24 h. The inactive anomer, β-GalCer (100 nM), was used as control. Two NKT tumor cell lines were then added to the DCs or splenocytes for 48 h, as potential responders to the presentation of αGalCer bound to CD1d. For the last 4 h, 3H-thymidine was added to monitor cell proliferation.

Results to date:

1. RA significantly reduced the proliferation of the two NKT tumor cell lines, DN32.2 and TCB11. Proliferation was also decreased in co-cultures of NKT cells with DC, but not with spleen cells.
2. Spleen cells secreted IL-4 and IFNγ only when cultured with αGalCer. The presence of the NKT tumor cells further increased spleen cell cytokine production, although the NKT cells alone produced neither cytokine.
3. The production of IL-4 and IFNγ by spleen cells was differentially regulated by RA, as RA increased the output of IL-4 when αGalCer-activated spleen cells were co-cultured with NKT cells, but at the same time RA reduced the output of IFNγ.

Conclusions: The growth-inhibitory effects of RA on the NKT tumor cells is encouraging, but further in vivo studies are needed. αGalCer markedly induced IFNγ production by splenocytes. The attenuation by RA needs to be further evaluated, especially with poly-I:C included in the triple stimulation model.

All-trans-Retinoic acid and α-galactosylceramide decrease the rate of lung metastasis in a murine 4T1 breast cancer model

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Abstract

All-trans-retinoic acid (RA) is a known regulator for cell growth and differentiation. It also plays critical roles in the regulation of immune response (1). αGalactosylceramide (αGalCer) is a glycolipid antigen that boosts immune system through CD1d-mediated NKT cell activity. Both RA and αGalCer are reported experimentally and clinically to be used in cancer treatment (2). As RA is a potent inducer of CD1d expression, current study focused on the effect of RA in αGalCer-mediated antitumor response. Mouse breast cancer 4T1 cells were injected into Balb/c mice intravenously. Animals were divide into 4 groups for treatment in the presence and absence of RA and/or αGalCer. αGalCer was given at 2 μg/mouse s.c. every other day and RA was given orally at a dose of 37.5 μg/mouse daily. After 12 days, lung and spleen tissues were collected. The size of spleen did not change significantly between the groups of 4T1 cell injected animals and the normal controls, however the CD11b$^+$Gr1$^+$ cell population dramatically increased after inoculation of 4T1 cells to the animals, about six times more than in uninjected control mice. Treatment of RA and/or αGalCer did not change the cell population very much among the 4T1 treated animals. A micrometastasis assay of the lung revealed that the number of metastatic foci was decreased in mice treated with RA and αGalCer. Further analysis of the lung showed that the expression level of matrix metalloproteinases (MMP) RNA, such as membrane type 1 (MT1)-MMP and MMP3, were increased after 4T1 cell inoculation, which was decreased in the presence of RA. Overall the results indicated that the combined treatment of RA and αGalCer can reduce the formation of lung metastatic foci. The regulation of MMP level may be one of the mechanisms to explain the regulatory effect of RA.

Materials and Methods

Animals and experimental design: 4T1 cells were injected to 8-weeks-old female Balb/c mice through retro-orbital sinus (2 x 10^4/mouse in 100 μl PBS) as a model of hematogenous tumor spreading. Twenty-four hours after inoculation, mice were randomized into four groups, vehicle, RA, αGalCer and RA plus αGalCer. Oral dose was given with RA (37.5 μg/mouse in canola oil) or vehicle for 10 days. αGalCer was injected subcutaneously every other day (2 μg/mouse, Enzo Life Sciences). Lung and spleen were collected for analysis at the end of the experiment, at day 14 of 4T1 cell inoculation.

Micrometastasis assay: Lung tissues were minced and digested with collagenase type IV (10 mg/ml) and elastase (10 u/ml), (Worthington Biochemical Corporation), for 30 minutes. Cells were washed and then cultured in 6-well plates with the medium containing 60 μM of 6-thioguanine (2). Colonies often formed after 7 to 10 days of culture. The colonies were then fixed with methanol and stained with 0.1% crystal violet to count the number of colonies in each well. The dye was then dissolved in 10% acetic acid to measure the absorbance at 570 nm.

Flow cytometric analysis: Splenocytes were prepared and stained with antibodies to CD19-PEcy7, CD3-FITC, Gr-1-FITC, and CD11b-PE (BD Biosciences).

Quantitative real-time PCR (qPCR): Lung tissues after culture were homogenized to extract total RNA. Reverse transcription was performed and followed by qPCR. 18S and Hypoxanthinephophoribosyltransferase (HPRT) were used as internal control to indicate the equality of the amplification. Also, 4T1 cells cultured for 24 hours were lysed and the total RNA was extracted for qPCR analysis. A SYBR green master mixture was used in the quantification of PCR.

ELISA to measure the MMP3 levels: MMP3 protein was measured for the plasma samples from the animals and the 4T1 cell culture medium by using an ELISA kit for mouse MMP3 (Abcam).

Statistic analysis: Anova was used in the Prism (Graphpad) software to compare the difference between each groups. $P < 0.05$ was considered significant.

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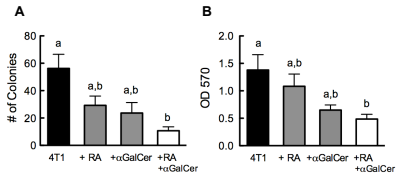
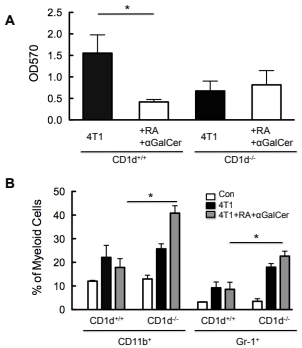
Figure 1. RA and αGalCer decrease lung metastasis after intravenous injection of 4T1 cells



Single cell suspensions of lung tissue after digestion were placed into culture, and the metastasis rate of 4T1 cells in lung was evaluated by determining the formation of 4T1 cell microfoci. The cells were stained with crystal violet and the the number of foci was counted under a microscope. The absorbance of dissolved crystal violet dye was also determined. RA plus αGalCer decreased the lung metastasis rate in micrometastasis assay shown by fewer number of colonies (**A**), and a lower level of crystal violet staining (**B**). a > b, $P < 0.05$.

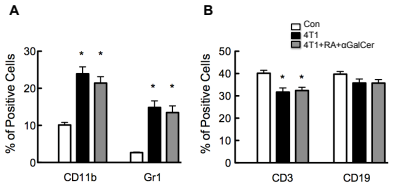
Figure 3. RA and αGalCer decrease the 4T1 tumor metastasis only in CD1d wild type mice



Wild and CD1d knockout mice were compared after 4T1 cell inoculation. **A**. Lung metastasis rate was decreased only in the wild type mice after RA and αGalCer treatment. **B**. Spleen myeloid cell population was increased to a higher level in the CD1d null mice. *, $P < 0.05$.

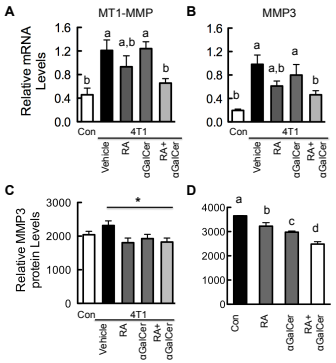
Results

Figure 2. Spleen myeloid cell populations are markedly increased after 4T1 cell inoculation



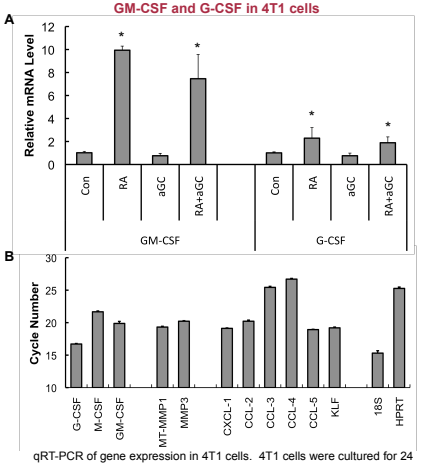
Splenocytes were prepared at the time of dissection and subjected to flow cytometry. CD11b$^+$ cells were markedly increased in 4T1 cell injected animals, especially Gr-1$^+$ cells (**A**), while at the same time, the T cell population (CD3$^+$) was decreased (**B**), and to a lesser extent the B cell population (CD19$^+$). * $P<0.05$. Treatment with RA plus αGalCer did not affect the 4T1 cell-induced spleen cell population change.

Figure 4. RA and αGalCer alter the expression levels of matrix metalloproteinase at both animal and cell models



RA plus αGalCer decreased the MT1-MMP and MMP3 mRNA levels of the lung tissue (**A and B**) and MMP3 protein level in the plasma (**C**) of the animals (a>b, *, $P < 0.05$). **D**. RA and αGalCer alone or in combination decreased the MMP3 protein level in the 4T1 cell culture supernatant (a>b>c>d, $P < 0.05$). 4T1 cells were cultured in the presence and absence of RA (20 nM) and αGalCer (100 ng/ml) for 24 hours. The supernatants were collected and subjected to ELISA.

Figure 5. RA increases the gene expression levels of GM-CSF and G-CSF in 4T1 cells



qRT-PCR of gene expression in 4T1 cells. 4T1 cells were cultured for 24 hours and the total RNA was extracted and subjected to qRT-PCR. **A**. 4T1 cells were cultured with and without RA (20 nM) and αGalCer (100 ng/ml) for 24 hours. GM-CSF and G-CSF gene expressions were measured. **B**. Data showing the expression level of each gene in 4T1 cells. Gene expression level was expressed as threshold cycle numbers, with the threshold set at the same level. *, $P < 0.05$.

Conclusions

- Combined treatment of mice with RA and αGalCer decreased 4T1 cell-induced lung metastasis. This was CD1d-dependent.
- RA and αGalCer treatment did not affect 4T1 cell induced leukemoid reaction, such as the increased myeloid cells in spleen.
- RA and αGalCer may decrease 4T1 cell-induced lung metastasis through the regulation of MMP expression.

Acknowledgment

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